JC17 Rec'd PCT/PTO 16 MAY 2001

FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **07121.0001U1**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5): 09/856025

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA99/01093	**NOVEMBER 16, 1999**	**NOVEMBER 16, 1998**

TITLE OF INVENTION
THERMOSTABLE XYLANASES

APPLICANT(S) FOR DO/EO/US
SUNG *et al.*

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

A Sequence Listing Diskette
A copy of the Written Opinion
A copy of the Article 34 Amendment
A copy of the Notification of the Recording of a Change
Return Postcard

 PCTUS1/REV03

EL348125745US

JC18 Rec'd PCT/PTO 16 MAY 2001

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/856025	PCT/CA99/01093	

21. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS	PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1,000.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO	**$860.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$710.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$690.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	32 - 20 =	12	x $18.00	**$216.00**	
Independent claims	4 - 3 =	1	x $80.00	**$80.00**	
Multiple Dependent Claims **(check if applicable).**			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,286.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).**			☐	**$0.00**	
SUBTOTAL =				**$1,286.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,286.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**			☐	**$0.00**	
TOTAL FEES ENCLOSED =				**$1,286.00**	
				Amount to be: refunded	$
				charged	$

☒ A check in the amount of **$1,286.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees. A duplicate copy of this sheet is enclosed.

☐ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

Gwendolyn D. Spratt

NAME

36,016

REGISTRATION NUMBER

5-16-01

DATE

CERTIFICATE OF EXPRESS MAILING

I hereby certify that the U.S. National Phase Application in the name of: Sung *et al.* consisting of: Transmittal Letter to the United States Designated/Elected Office (DO/EO/US) Concerning a Filing Under 35 U.S.C. 371 PTO Form 1390 (2 Pages); a copy of the as-filed International Application (38 pages/specification; 3 pages/claims; 12 pages formal drawings and 28 pages Sequence Listing); a Sequence Listing Diskette; a copy of the Written Opinion; a copy of the Article 34 Amendment; a copy of the International Search Report; a copy of the International Preliminary Examination Report; a copy of the Notification of the Recording of a Change; a check in the amount of $1,286.00 to cover filing fees and a return postcard for "**THERMOSTABLE XYLANASES**" is being deposited with the United States Postal Service as Express Mailing No. EL348125745US in an envelope addressed to: Assistant Commissioner for Patents, Box PCT (IPEA/EP), Washington, D.C. 20231, on this **16th** day of **May 2001**.

EVERARDO MCFARLANE

5-16-01
Date

W098453

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THERMOSTABLE XYLANASES

The present invention relates to thermostable xylanase enzymes. More specifically, the present invention is directed to thermostable xylanase enzymes that exhibit high activity at or near physiological pH and temperature, and their use in feed pelleting applications.

BACKGROUND OF THE INVENTION

Natural xylanase enzymes, such as that of the fungus *Trichoderma reesei*, have been added to animal feed to increase the efficiency of digestion and assimilation of nutrients. During digestion of feed grains such as wheat and barley, non-starch polysaccharides, including xylan, increases the viscosity of the digesta in the absence of added exogenous enzyme. This interferes with the diffusion of the digestive enzymes to the feed and the subsequent assimilation of the nutrients. The highly viscous digesta increases the occurrence of sticky stool, which increases the likelihood of disease and causes effluent run-off problems. The addition of xylanase in feed breaks down the xylan and decreases the viscosity of the digesta, thereby helping tp alleviate these problems. Xylanase produces a cost saving by increasing the efficiency of feed conversion. Xylanase can decrease the feed consumed/ weight gain ratio by 5-15% (Viveros, A., Brenes, A., Pizarro, M. and Castano, M., 1994, Animal Feed Sci. Technol. 48:237-251).

Xylanase enzymes used for feed are typically aqueous solutions of active protein, stabilizers, preservatives and other additives. The enzymes are typically sprayed onto the feed at concentration of 100-2000 ml per tonne feed. Alternatively, granular or powdered xylanase can be used. Once the feed is consumed by the animal, the enzyme acts on xylan as the feed is ingested and digested in the gut. Eventually the xylanase, a protein molecule, is hydrolysed by the digestive enzymes (proteases) into amino acids like any protein in the feed.

Increasingly, animal feeds are pelleted at high temperatures for sterilization against harmful bacteria, for example *Salmonella*. Feed pelleting is carried out by heating the feed solids with 100 to 140°C steam and passing them through an extruder/pelleting auger to form feed pellets, which then cool in a storage bin. The typical time required for the material to pass through the system is 30 minutes. As is known in the art, higher temperatures can be used with shorter pelleting times, and lower temperatures with longer pelleting times, provided that the necessary moisture levels are obtained. The overall resulting temperature within the solids, prior to, during, and after pellet formation reaches about 70-95°C, for up to 30 min. It is desirable to add the xylanase during the feed pelleting process. This would save the feed formulators the additional step of adding liquid xylanase, which is inconvenient and can introduce microbial contamination into the feed. The option of adding solid xylanase as a separate step is also undesirable, as the solids would not be evenly mixed. Marquardt and Bedford (1997, Enzymes in Poultry and Swine Nutrition, Marquardt R.R. and Han Z. eds., pp.129-138) indicate that even though currently available enzymes are beneficial for use as feed additives, new enzymes exhibiting high activity and resistance to heat treatment are also desired, however. they note that enzymes exhibiting these properties are not available.

Xylanases of Family 11 (also termed Family G xylanases) have several properties suitable for feed applications due to their small size and high activity. An example of a moderate temperature Family 11 xylanases is TrX, which is obtained from *Trichoderma reesei*. Moderate temperature xylanases are proven feed additive enzymes with temperature and pH optima compatible with the physiological conditions in the digestive system of animals. However, these enzymes can not tolerate the high temperature of the pelleting process and become inactive during this step.

Xylanases from high temperature microorganisms (eg. a thermophile), for example *Thermomonospora fusca* xylanase (termed TfX, also a Family 11 xylanase), have also been considered for feed pelleting. The thermostability of such enzymes is sufficient to tolerate the pelleting temperatures. However, thermophilic xylanases have

optimum activity at high temperatures (70-80°C), and several of these enzymes have a high pH optimum of 7-9. When introduced into the digesting system of an animal, with a physiological temperature of around 40°C (e.g. poultry 43°C, a similar temperature is noted within swine) and pH of 3-5 in the digesta, these enzymes function poorly.

Family 11 xylanases have been modified by protein engineering to improve the properties of these enzymes for industrial applications. These modifications have been directed at increasing the temperature and pH optima, along with the thermostability, of these enzymes for specific applications. For example, US 5,405,769 (WO 94/24270) is directed to site-specific mutagenesis of *Bacillus circulans* xylanase (BcX) for the improvement of the thermostability of this enzyme. The disclosed modifications relate to the formation of intermolecular and intramolecular disulfide bonds within BcX, and these modifications resulted in increased thermostability. For example, an improvement in thermostability of up to 6°C with the addition of a single disulfide bond, and up to 10°C with two disulphide bonds was observed. Other modifications included linking the N- and C- termini which increased thermostability by 6°C, or N-terminal mutations, which increased thermostability by 2°C. However, with all of the above modifications the resultant enzymes were either less active (up to 45% less active), or exhibited an increase in the temperature and pH optima. As such these enzymes are not suitable for feed pelleting applications.

US 5,759,840 also discloses modifications to BcX and *Trichoderma reesei* xylanase (TrX) to increase the thermostability; while at the same time increase the temperature and pH optima of these enzymes. Again, these xylanases would not be suitable for feed pelleting applications.

The above results are in agreement with other reports that note that disulfide bonds are not among the thermostabilization mechanisms employed by thermophilic enzymes (Cowan, D.A., 1995, Essays Biochem. 29:193-207), as the disulfide can be broken into dehydroalanine and thiocysteine at temperatures over 80°C. Therefore, the

enhancement of stability of an enzyme using disulfide bonds is limited to lower temperature ranges. The disulfide bond is thus not recommended to improve the stability of the enzyme at high temperatures (Gupta, M.N., 1991, Biotech. Applied Biochem. 14:1-11; Cowan, D.A., 1995, Essays Biochem. 29:193-207.).

None of the above documents address methods for obtaining xylanase enzymes using conventional screening techniques, or by modifying xylanase enzymes, that exhibit the properties of higher temperature tolerance while maintaining optimal performance under conditions of physiological pH and temperature.

An improvement in the thermostability of *Trichoderma reesei* xylanase II was reported by Paloheimo et al (Paloheimo, M., Mantyla, A., Vehmaanpera, J., Hakola, S., Lantto, R., Lahtinen, T., Parkkinen, E., Fagerstrom, R. and Suominen, P. 1997, *in* Carbohydrases from Trichoderma reesei and Other Microorganisms p255-264). Of the five mutants characterized, the most improved mutant (glutamic acid-38 TrX) retained 50% of activity at 57°C after 9 min, as compared to 7 min by wide type TrX. Arase et al (Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y. and Okada, H., 1993, FEBS Lett. 316:123-127) describes several modifications to improve the thermostability of a *Bacillus pumilis* xylanase (BpX), however only up to 40% of the residual enzymatic activity was maintained following incubation of these enzymes at a temperature of 57°C for 20 min. Even though, in both of these studies the effects of increased thermostability on pH and temperature optima of the enzymes were not determined, these enzymes exhibit inadequate thermostability for feed pelleting applications.

In spite of a wide range of experience in screening, testing and modifying xylanase enzymes, there are no reports of xylanases that exhibit the combination of properties required for feed pelleting applications: high thermostability, with optimum activity at physiological pH and temperature. No natural xylanases have been selected, nor has any mutation methodology for the Family 11 xylanases been developed to increase thermostability of xylanase enzymes to, without any change in the temperature

and pH optima, and a concomitant loss of the specific activity of the enzyme. Such selected natural xylanases, or xylanases prepared using mutation methodology would offer the advantages of enhancement of feed digestibility and processing in pelleting.

The present invention is directed to obtaining xylanase enzymes that exhibit the property of increased thermostability, while maintaining pH and temperature optima that are typically found under physiological conditions.

It is an object of the invention to overcome disadvantages of the prior art.

The above object is met by the combinations of features of the main claims, the sub-claims disclose further advantageous embodiments of the invention.

SUMMARY OF THE INVENTION

The present invention relates to thermostable xylanase enzymes. More specifically, the present invention is directed to thermostable xylanase enzymes that exhibit high activity at or near physiological pH and temperature, and the use of these xylanase enzymes in feed pelleting applications.

According to the present invention there is provided an isolated xylanase comprising at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 60°C, the isolated xylanase being thermostable. The thermostability is characterized by the isolated xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 70°C in the presence of 40% glycerol. The thermostability may also be characterized by the isolated xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 62.5°C.

The present invention is also directed to a modified xylanase, comprising at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40

to about 60°C, the modified xylanase being thermostable. This invention also embraces a modified xylanase comprising a basic amino acid at position 162 (TrX numbering), or its equivalent position. The basic amino acid is selected from the group consisting of lysine, arginine and histidine. Preferably the basic amino acid is histidine.

This invention also pertains to the modified xylanase as defined above, wherein the modified xylanase comprises at least one disulfide bridge. Preferably, the modified xylanase comprises one or two disulfide bridges.

The present invention is also directed to a modified xylanase as defined above, wherein the xylanase is a Family 11 xylanase. Furthermore, this invention pertains to a modified xylanase, wherein the Family 11 xylanase is from *Trichoderma*.

The present invention is also directed to the modified xylanase as defined above wherein said xylanase is selected from the group consisting of TrX-162H-DS1, TrX-162H-DS2, and TrX-162H-DS4.

This invention also includes a method of obtaining a xylanase comprising:

i) selecting an organism that exhibits xylanase activity, obtaining xylanase from the organism;
ii) determining whether the xylanase exhibits at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 60°C;
iii) determining whether the xylanase is thermostable; and
iv) retaining the xylanase that express these properties

Step i) of the above method may also include partially purifying the xylanase.

The present invention also pertains to a method of preparing animal feed, wherein the method comprises applying the isolated xylanase as defined above onto the

animal feed to produce a xylanase-animal feed combination, and heat sterilizing the xylanase-animal feed combination. Preferably, the animal feed is a poultry or swine feed.

The present invention is directed to obtaining xylanase enzymes that exhibit pH and temperature optima that are found within the digesta of an animal, while at the same time the xylanase molecule exhibits thermostability and can therefore withstand processes associated with sterilizing and producing pelleted feed. The prior art discloses obtaining thermostable enzymes, either through selection of native enzymes, or through genetic engineering, however, these enzymes do not exhibit physiological pH and temperature optima. The prior art also discloses xylanase enzymes that exhibit optimal enzyme activity at physiological pH and temperature, however, these enzymes are not thermally stable. Furthermore, there is nothing in the prior art to suggest that native xylanase enzymes exist, or that xylanase enzymes may be modified as disclosed herein win order to obtain xylanase enzymes that exhibit high temperature tolerance suitable for feed pelleting, and retain optimum enzymatic activity at or near physiological conditions.

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a sub-combination of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

FIGURE 1 shows the multiple amino acid sequence alignment among family 11 xylanases. The amino acids common to at least 80% of the Family 11 xylanases listed are indicated in bold. The residues common to all Family 11 xylanases are underlined. *Bacillus pumilus* (Bp); *Clostridium acetobutylicum* P262 XynB

(Ca); *Clostridium stercorarium* (Cs); *Ruminococcus flavefaciens* (Rf); *Trichoderma reesei* XynII (Tr2); *Trichoderma viride* (Tv); *Trichoderma harzianum* (Th); *Schizophyllum commune* Xylanase A (Sc); *Aspergillus niger* var. awamori (An); *Aspergillus tubigensis* (At); *Trichoderma reesei* XynI (Tr1); *Streptomyces sp. No. 36a* (Ss); *Streptomyces lividans* Xylanase B (S1B); *Streptomyces lividans* Xln C (S1C); *Thermomonospora fusca* TfxA (Tf); *Bacillus circulans* (Bc); *Bacillus subtilis* (Bs)

FIGURE 2 shows the synthetic oligonucleotides for the construction of gene sequence encoding the *Trichoderma* xylanase in the plasmid pTrX (SEQ ID NO:18).

FIGURE 3 shows the effect of incubation time on the residual enzymatic activity of mutant TrX. TrX-DS1, TrX-162H, TrX-162H-DS1, and TrX-162H-DS4 at 62.5°C. The data are normalized to that observed at 0 min.

FIGURE 4 shows the effect of temperatures on the residual enzymatic activity of several of the modified xylanases of the present invention. Figure 4(a) shows the residual enzymatic activity of TrX, TrX-DS1, TrX-162H-DS1, TrX-162H-DS2, and TrX-162H-DS4 in sodium citrate buffer in a 30 min incubation. Figure 4(b) shows the effect of temperatures on the residual enzymatic activity of the mutant TrX-DS8. For Figures 4(a) and (b) The data are normalized to that observed at 48°C. The T_{50}, which is the incubation temperature allowing the maintenance of 50% residual activity after 30 min, was determined for each mutant TrX.

FIGURE 5 shows the effect of temperatures on the residual enzymatic activity of mutant TrX, Trx-DS1 and TrX-162H-DS1 in 40% glycerol in a 30 min incubation. The data are normalized to that observed at 50°C.

FIGURE 6 shows the effect of incubation time on the residual enzymatic activity of TrX-162H-DS1 in 40% glycerol at 90°C. The data are normalized to that observed at 0 min.

FIGURE 7 shows the effect of temperature on release of xylose in a 30 min hydrolysis of soluble xylan by TrX, TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4 at pH 4.5. The data are normalized to that observed at the temperature optimum.

FIGURE 8 shows the effect of pH on the release of xylose in a 7 min hydrolysis of soluble xylan by TrX, TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4 at 40°C. The data are normalized to that observed at the pH optimum.

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to thermostable xylanase enzymes and their use as feed additives. More specifically, the present invention is directed to thermostable xylanase enzymes that show good thermostability and exhibit high activity at or near physiological pH and temperature.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

By physiological pH and temperature, it is meant the range in temperature and pH compatible with the digestive system within an animal, for example but not limited to, poultry and swine. For example, a suitable physiological temperature range is from about 35 to about 60°C, more preferably, this range is from about 40 to about 50°C. Similarly, a suitable physiological pH range is from about pH 3.0 to about 7.0, preferably, this range is from about pH 3.5 to about 6.0. The time required for the digestion of feed within the gut of an animal varies from animal to animal. For example, in swine digestion of feed is from about 2 to about 4 hours, while in poultry it is up to about 12 hours.

By high activity at physiological pH and temperature, it is meant that the enzyme exhibits at least 40% of its optimum activity at physiological pH and temperature. The optimum pH and temperature range can be outside the physiological range, provided that the enzyme exhibits at least 40% of its optimum activity within the physiological range, for example from about 40 to about 50°C and pH from about 3.5 to about 6. Examples 4 and 5 describe the determination of a suitable xylanase enzyme that exhibits these properties.

"Thermostable" or "thermostability" as used herein refer to a property of an enzyme. An enzyme is considered to be thermostable if it exhibits at least one of the following properties:

1) the enzyme exhibits at least 30% of its optimal activity following a pre-incubation step of 30 min at 70°C, 80°C, or 90°C, at pH 5.0, in the presence of a stabilizing agent such as 40% glycerol. Preferably, the enzyme exhibits at least 40% of its optimal activity following a 30 min, 70°C pre-incubation step in glycerol, for example but not limited to, TrX-162H-DS1 (Figure 5);

2) the enzyme exhibits 30% of its optimal activity following a pre-incubation step of 30 or 60 min at 62.5°C in the absence of a stabilizer. Preferably, the enzyme exhibits at least 40% of its optimal activity following a 30 min pre-incubation, for example but not limited to, TrX-162H-DS1 and TrX-162H-DS4 (Figure 3);

3) the enzyme exhibits at least 30% of its optimal activity following a preincubation step of 30 min at 64°C in the absence of a stabilizer. Preferably, the enzyme exhibits at least 40% of its optimal activity following the 30 min, 64^0C pre-incubation step, for example but not limited to, TrX-162H-DS1 and TrX-162H-DS4 (Figure 4); or

4) the enzyme exhibits at least 30% of its optimal activity following a preincubation step of 30 min at 68°C in the absence of a stabilizer. Preferably, the enzyme exhibits at least 40% of its optimal activity following the 30 min, 68°C pre-incubation step, for example but not limited to, TrX-162H-DS1 and TrX-162H-DS4 (Figure 4).

In each of the above cases, the optimal activity of the enzyme is determined at an optimal pH and temperature for that enzyme in the presence or absence of stabilizer as required.

By "TrX numbering" it is meant the numbering associated with the position of amino acids based on the amino acid sequence of TrX (Xyn II - Table 1; Tr2 - Figure 1). As disclosed below and as is evident upon review of Figure 1, Family 11 xylanases exhibit a substantial degree of sequence homology. Therefore, by aligning the amino acids to optimize the sequence similarity between xylanase enzymes and by using the amino acid numbering of TrX as the basis for numbering, the positions of amino acids within other xylanase enzymes can be determined relative to TrX.

By modified xylanase, it is meant the alteration of a xylanase molecule using techniques that are known to one of skill in the art. These techniques include, but are not limited to, site directed mutagenesis, cassette mutagenesis, synthetic oligonucleotide construction, cloning and other genetic engineering techniques. Alterations of a xylanase enzyme, in order to produce a modified xylanase may also arise as a result of applying techniques directed at inducing mutations within native or genetically engineered xylanases via the addition of known chemical mutagens, UV exposure, or other treatments known to induce mutagensis within a host organisms that express a xylanase of interest. Such techniques are well known within the art.

Table 1 lists the Family 11 xylanases free of cellulase activity. These enzymes share extensive amino acid sequence similarity and possess amino acids common to Family 11, for example two glutamic acid (E) residues serving as the essential catalytic residues, amino acids 86 and 177 (using TrX numbering). Structural comparisons of several Family 11 xylanases via X-ray crystallography indicates that these Family 11 xylanases of bacterial and fungal origins share the same general molecular structure (see for example US 5,405,769; Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y. and Okada, H., 1993, FEBS Lett. 316:123-127). Most of the family 11 xylanases identified so far are mesophilic and have low-molecular mass (20kDa).

TABLE 1: Family 11 xylanases

Microbe	Xylanase	Ref. in Figure 1	Sequence Listing
Aspergillus niger	Xyn A	An	SEQ ID NO: 1
Aspergillus kawachii	Xyn C		
Aspergillus tubigensis	Xyn A	At	SEQ ID NO: 2
Bacillus circulans	Xyn A	Bc	SEQ ID NO: 3
Bacillus pumilus	Xyn A	Bp	SEQ ID NO: 4
Bacillus subtilis	Xyn A	Bs	SEQ ID NO: 5
Cellulomonas fimi	Xyn D		
Chainia spp.	Xyn		
Clostridium acetobutylicum	Xyn B	Ca	SEQ ID NO: 6
Clostridium stercorarium	Xyn A	Cs	SEQ ID NO: 7
Fibrobacter succinognees	Xyn C		
Neocallimasterix patriciarum	Xyn A		
Nocardiopsis dassonvillei	Xyn II		
Ruminococcus flavefaciens	Xyn A	Rf	SEQ ID NO: 8
Schizophyllum commune	Xyn	Sc	SEQ ID NO: 9
Streptomyces lividans	Xyn B	S1B	SEQ ID NO: 10
Streptomyces lividans	XynC	S1C	SEQ ID NO: 11
Streptomyces sp. No. 36a	Xyn	Ss	SEQ ID NO: 12
Streptomyces thermoviolaceus	XynII		
Thermomonospora fusca	Xyn A	Tf	SEQ ID NO: 13
Trichoderma harzianum	Xyn	Th	SEQ ID NO: 14
Trichoderma reesei	Xyn I	Tr1	SEQ ID NO: 15
Trichoderma reesei	Xyn II	Tr2	SEQ ID NO: 16
Trichoderma viride	Xyn	Tv	SEQ ID NO: 17

It is considered within the scope of the present invention that xylanases, including Family 11 xylanases for example but not limited to *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase I, *Trichoderma viride* xylanase, *Streptomyces lividans* xylanase B and *Streptomyces lividans* xylanase C, may be modified following the general approach and methodology as outlined herein. It is also considered within the scope of the present invention that non-Family 11 xylanases may also be modified following the general principles as described herein in order to obtain a xylanase enzyme that is thermostable and exhibits high activity at physiological pH and temperature.

Furthermore, native xylanases may also be obtained by using standard screening protocols in order to identify enzymes that exhibit the properties of increased thermostability yet maintaining high activity at physiological temperature and pH. Such protocols involve:

- selecting of a desired organism, for example a thermophile;
- extracting or obtaining the xylanase from the organism. and partially purifying the enzyme if desired; and
- characterizing the extracted enzyme to determine whether the enzyme is thermostable, as defined above (in the presence or absence of a stabilizing agent, such as glycerol), determining the enzymes pH and temperature optima, and determining the activity of the enzyme at physiological pH and temperature.

Any enzymes identified using the above protocol that exhibit thermostability and high activity at physiological pH and temperature may be used as animal feeds.

The present invention also relates to modified xylanase enzymes that exhibit increased thermostability while maintaining high activity at physiological pH and temperature. For example, and without wishing to limit the present invention in any manner, a modified *Trichoderma reesei* xylanase (TrX) is disclosed that exhibits increased thermostability while maintaining pH and temperature optima at or near physiological range. Two modifications in the TrX were combined in order to obtain a novel xylanase (TrX-162H-DS1). The first modification includes a double mutation to create two cysteines for the formation of a single disulfide bond. Such a modification has been described for *Bacillus circulans* xylanase (C100/C148; BcX amino acid numbering) in US 5,405,769. However, this mutation bestows only a minor increase in the ability of the enzyme to withstand high temperatures (see TrX-DS1, Figures 3-5) and this modification is not adequate to produce an enzyme capable of surviving high temperatures associated with the pelleting process. When this mutation is combined with a second mutation as per the teaching of this invention, involving the substitution of a basic amino acid such as histidine (H) for glutamine (Q) in position 162, the resultant combination mutant xylanase exhibits the desired properties of thermostability (TrX-162H-DS1; see Figures 5 and 6), and greater than 40% of optimum activity at physiological pH (Figure 8), and temperature (Figure 7).

Another mutant xylanase in the present invention, TrX-162H-DS4 differs from TrX-162H-DS1 by possessing an additional disulfide (108/158, that is between positions 108 and 158). This type of double disulfide mutant has previously been described for the xylanase of *Bacillus circulans* (C98/C152, 100/148; BcX amino acid numbering; Wakarchuck et al . 1994 Protein Engineering, 7:1379-1386). The BcX mutant does not comprise an equivalent basic amino acid (e.g. H for Q at position 162) substitution as disclosed herein. The mutant TrX-162H-DS4 shows a dramatic increase of thermostability (see Figure 4(a)), with an increase in the T_{50} of TrX-162H-DS4 of 14°C. This is an improvement over the prior art double disulfide BcX mutant which exhibits an increase in the T_{50} of 10°C, thereby demonstrating the contribution of the Q162H mutation in the disulfide mutants of TrX.

The present invention also pertains to additional mutations that have been found to be effective in producing a xylanase that exhibits thermostability and a desirable pH profile. An example of such mutations may be found in, but are not limited to, TrX-DS8. TrX-DS8 includes the mutations listed for N1-TX13 as disclosed in US 5,759,840, namely N10H, Y27M and N29L, and also includes N44D, Q125A, I129E, Q162H and a disulfide bond between positions 110 and 154. Trx-DS8 exhibits the property of thermostability (Figure 4(b)), a pH profile parallelling that of TrX-162-DS1, and greater than 40% of optimum activity at physiological pH, and temperature.

Xylanase enzymes comprising the substitution of H for Q at position 162 (termed Q162H) in isolation has been reported in US 5,759,840, however, these mutants exhibited no improvement in thermostability or other properties over natural TrX. However, by combining these two modifications, several novel xylanases (TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4) were obtained with improved thermostability. This property was not observed with either mutation alone. Furthermore, these modified xylanases exhibit high activity at or near physiological temperature and pH. These mutations are also found in Trx-DS8, which also exhibits improved thermostability and high activity at or near physiological conditions.

Following the methods of the present invention novel xylanase enzymes may be obtained that are far more suitable for feed pelleting applications than enzymes currently available. Similar modifications may be made in other Family 11 xylanases, including but not limited to, xylanase enzymes obtained from *Trichoderma*, *Streptomyces* and *Schizophyllum*. However, it is also within the scope of the present invention that other xylanase enzymes, in addition to Family 11 xylanases can be modified as disclosed herein in order to obtain xylanases with that are thermostable and exhibit high activity at physiological pH and temperature. Furthermore, it is within the scope of the present invention that native xylanase enzymes with the properties of thermostability and high activity at physiological pH and temperature may be obtained following screening protocols that select for both thermostability and high activity at physiological pH and temperature.

In use, the formulation of the feed enzyme can improve the enzymes thermostability, as adsorption into feed improves stability as the enzyme is brought into contact with its substrate. Therefore, in determining thermostability of the xylanases of the present invention, xylanases were characterized in the presence and absence of stabilizing agents, for example but not limited to glycerol. Fisk and Simpson (1993) have reported that 40% glycerol enhanced the temperature tolerance of wild type TrX by less than +10°C, however, this is much less stability than the enzymes of the present invention. The combination-mutant xylanases of the present invention can tolerate incubation in buffer at a higher temperature (59-69°C), as compared to natural xylanase (55°C; also see Figure 3 and 4). In the presence of 40% glycerol, the combination mutants can retain a substantial portion of their activity at 70 to 90°C (see figure 5), while the natural xylanase is totally inactivated at these temperatures..

One of the modifications to the combination mutant xylanase as proposed herein is the substitution of amino acid 162 (TrX numbering, based on Tr2 in Figure 1; which for TrX is glutamine) with the basic amino acid histidine (termed Q162H). However, it is considered within the scope of the present invention that other amino acids may also be substituted at this position. Preferably the substituted amino acid is basic (positively charged), for example lysine (Q162K) or arginine (Q162R). It has been observed herein that the substitution at the position 162, or its equivalent in other Family 11 xylanases, by a basic amino acid such as histidine can greatly improve the thermostability of a xylanase enzyme that comprises at least one intramolecular disulfide bond. Importantly, it has also been observed herein that this substitution at position 162 not only increases thermostability but also does not significantly change the temperature and pH profiles, and the specific activity of the modified xylanase.

Histidine-162 residue (TrX numbering) in the combination mutant is found in several natural Family 11 xylanases, such as those of *Trichoderma harzianum*, *Aspergillus niger*, *var. awamori*, *Aspergillus tubigensis*, *Thermomonospora fusca*, *Bacillus circulans* and *Bacillus subtilis* in the corresponding position. Similarly,

Clostridium acetobutylicum comprises a lysine at this equivalent position. However, all, of these xylanases, with the exception of the *Thermomonospora fusca* xylanase, are produced by mesophilic hosts and exhibit low thermostability. As a result there is no evidence to suggest any beneficial effect on thermostability by presence of a basic amino acid residue at this position. In the *Thermomonospora fusca* xylanase, the N-terminal sequence (1-29) which is distant from the site of the present invention, has been shown to contribute to thermostability, and there is no evidence to suggest that thermostability may be associated with a histidine at this equivalent position (i.e. TrX 162).

This invention is also directed to xylanases that comprise at least one modification that results in increased thermostability while maintaining high activity at physiological pH and temperature. For example, native *Schizophyllum commune* xylanase has a disulfide bond at positions 110/154 (TrX numbering). However, this enzyme exhibits low thermostability, Therefore, this enzyme can be modified using the methods of the present invention to substitute a basic amino acid, either histidine, arginine or lysine for the naturally occurring leucine at position 200 of *Schizophyllum commune* (which is equivalent to position 162 using TrX numbering; see Figure 1; Sc). Therefore, increased thermostability can be achieved through a one-step modification.

Also considered within the scope of the present invention are combination mutants comprising both an intramolecular disulfide bond and a basic amino acid substitution as outlined above. The intramolecular disulfide bond may arise as a result of a mutation at one or more specific residues, for example (per TrX numbering):

- residues-110/-154, for example, but not limited to TrX-162H-DS1 or Trx-DS8;
- residues-108/-158, for example, but not limited to TrX-162H-DS2; or
- residues-108/-158, -110/-154, for example, but not limited to TrX-162H-DS4.

Also considered within the scope of the present invention are modifications of thermostable xylanases, for example, but not limited to TfX. These modifications maintain the thermostability of the native enzyme, yet alter the pH and temperature optima so that they exhibit high activity at physiological pH and temperature not normally associated with the enzyme.

TABLE 2: Modified xylanases

XYLANASE	DESCRIPTION
wild type TrX	wild type *T. reesei* xylanase.
TrX-162H	TrX mutant with mutation Q162H.
TrX-DS1	TrX mutant with an intramolecular disulfide bond between positions-110 and 154.
TrX-162H-DS1	TrX mutant with two mutations, (i) a disulfide bond between positions-110 and 154, and (ii) mutation Q162H.
TrX-162H-DS2	TrX mutant with two mutations, (i) an intramolecular disulfide bond between positions-108 and 158, and (ii) mutation Q162H.
TrX-162H-DS4	TrX mutant with two mutations, (i) two intramolecular disulfide bonds at residues-110/154 and residues-108/158, and (ii) mutation Q162H.
TrX-DS8	Trx mutant with i) an intramolecular disulfide bond between positions-110 and 154, and ii) comprising mutations N10H, Y27M, N29L, N44D, Q125A, I129E, and Q162H

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

Examples:

Example 1: Construction of the *Trichoderma reesei* mutant xylanases

Basic recombinant DNA methods like plasmid preparation, restriction enzyme digestion, polymerase chain reaction, oligonucleotide phosphorylation, ligation, transformation and DNA hybridization were performed according to well-established protocols familiar to those skilled in the art (Sung, W. L., Yao, F.-L., Zahab, D. M. and Narang, S. A. (1986) Proc. Natl. Acad. Sci. USA 83:561-565) or as recommended by the manufacturer of the enzymes or kit. The buffer for many enzymes have been supplied as part of a kit or constituted following to the instruction of the manufacturers. Restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase were purchased from New England BioLabs LTD, Mississauga, Ont. A precursor plasmid pXYbc has previously prepared and published (Sung, W. L., Luk, C. K., Zahab, D. M. and Wakarchuk, W. (1993) Protein Expression Purif. 4:200-206; US 5,405,769). A commonly used E. coli strain, HB101 (clonetech Lab, Palo Alto, CA) was used as transformation and expression host for all gene construct. Birchwood xylan was purchased from Sigma (St. Louis, Mo). Hydroxybenzoic acid hydrazide (HBAH) was purchased from Aldricht. Oligonucleotides were prepared with an Applied Biosystem DNA synthesizer, model 380B. Xylanase assays have been performed in a covered circulating water bath (Haake type F 4391) with a fluctuation of "0.1°C. Temperature of the water bath was confirmed with a thermocouple.

A. Construction of the precursor plasmid pTrX

The precursor plasmid pTrX for all subsequent mutations is published (Sung et al, 1995). This plasmid is derived from a pUC119 plasmid with a synthetic nucleotide sequence encoding a *Trichoderma reesei* xylanase inserted (Figure 2). Expression of this xylanase and other mutant xylanases subsequently described are

under the control of the *lac* promoter of the pUC plasmid. The total assembly of the gene required two stages, initially for the (92-190) region, then followed by the (1-92) region. The protocol for the construction of this gene is routine and identical to the standard published procedure for many other genes. It required enzymatic phosphorylation of overlapping synthetic oligonucleotides which encodes xylanase. This was followed by their ligation into a appropriately cut plasmid pUC119.

Initially ten overlapping oligonucleotides:

XyTv-101,	SEQ ID NO:28
XyTv-102,	SEQ ID NO:29
TrX-103,	SEQ ID NO:30
XyTv-104,	SEQ ID NO:31
XyTv-105,	SEQ ID NO:32
XyTv-106,	SEQ ID NO:33
XyTv-107,	SEQ ID NO:34
TrX-108,	SEQ ID NO:35
XyTv-109,	SEQ ID NO:22
XyTv-110,	SEQ ID NO:36

encoding the TrX(92-190) sequence (Figure 2), were designed with codon usage frequency imitating that of *E. coli* (Chen et al. 1982). The SalI and BglII cohesive ends of two terminal oligonucleotides enabled the enzymatic ligation of the ten fragments to the linearized plasmid pXYbc. The ten oligonucleotides (50 pmol, 1 L for each) encoding the TrX(92-190) was phosphorylated in a mixture containing 10X standard kinase buffer (0.4 L), 1mM ATP (4 L), T4 DNA kinase (5 units), and water (3 L). Phosphorylation reaction was carried out for 1 h at 37°C. The solutions were then combined and heated to 70°C for 10 min. After being cooled slowly to room

temperature, the combined solutions were added to a mixture of 4mM ATP (3.5 L), EcoR1-HindIII linearized plasmid pUC119 (0.1 pmol), and T4 DNA ligase (3.5 L) and incubated at 12°C for 20 h. Aliquots of the ligation mixture were used to transform *E. coli* HB101 in YT plate (8 g yeast extract, 5 g bacto-tryptone, 5 g NaCl, 15 g of agar in 1 L of water) containing ampicillin (100 mg/L).

For the preparation of a hybridization probe, one of the oligonucleotide XyTv-110 (10 pmol, 1 L) was phosphorylated ^{32}P-ATP (10 pmol, 3 L) in T4 DNA kinase (1 L), 10X kinase buffer (1 L), and water (4 L) at 37°C for 1 h.

Transformants were selected randomly for hybridization analysis. Colonies were grown on nylon filters on YT plates with ampicillin overnight. They were then denatured with 0.5N NaOH - 1.5M NaCl (10 min) and neutralized with 0.5N Tris-HCl (pH 7.0) - 1.5M NaCl (10 min). After irradiation by UV of 254 nm for 8 min, the filters were washed with 6X SSC - 0.05% Triton X-100 for 30 min. Cell debris was scraped off completely. After another 30 min. in fresh solution, the duplicate filters were transferred individually into separate mixtures of 6X SSC - 1% dextran sulphate - 0.05% TritonX-100 - 1X Denhardt's hybridization fluid. The ^{32}P-labelled probe was added to the filter. After 16 h at 45°C, the filter was washed twice with 6X SSC - 0.05% TritonX-100 at room temperature for 5 min. and then at 65°C for 30 min. Positively hybridized clones with the intermediate plasmid pBcX.TrX were identified by auto-radiographic analysis.

The above protocol, involving enzymatic phosphorylation of synthetic overlapping oligonucleotides and ligation into a linearized plasmid, has again been used in the assembly of the TrX(1-92) region and in the cassette mutagenesis for the subsequent generation of other mutant series described in this invention.

For the assembly of the TrX(1-92) region to complete the full-length *Trichoderma* gene, the intermediate plasmid pBcX.TrX was linearized by NheI and KpnI endonucleases to release the DNA insert for BcX(1-83). With NheI and KpnI cohesive ends, eight overlapping oligonucleotides:

TrX-1,	SEQ ID NO:37
XyTv-2,	SEQ ID NO:38
TrX-3,	SEQ ID NO:39
XyTv-4,	SEQ ID NO:40
XyTv-5,	SEQ ID NO:41
TrX-6,	SEQ ID NO:42
XyTv-7,	SEQ ID NO:43
TrX-8,	SEQ ID NO:44,

encoding the published TrX(1-91) sequence were ligated into the linearized plasmid pBcX.TrX (Figure 2), via the protocol described above. The new plasmid pTrX therefore harbored a synthetic TrX gene (SEQ ID NO: 18).

All mutant xylanases described below have been constructed via the method of cassette mutagenesis as described above. The protocol for the cassette mutagenesis was identical to that for gene assembly fully described above. Such cassette mutagenesis involved (i) enzymatic phosphorylation of overlapping synthetic oligonucleotides, (ii) their ligation with the linearized plasmid, (iii) transformation into the *E. coli* HB101 competent cells, (iv) identification of the mutant transformants via hybridization with the labelled oligonucleotide as probe, and (v) confirmation of the mutation through dideoxy nucleotide sequencing.

B. Construction of the plasmid pTrX-DS1

The mutant TrX-DS1 (SEQ ID NO's:54, 55) was identical to TrX with a covalent disulfide bond between residues-110 and 154. This was accomplished through two single mutations, ie. conversion of both residues serine-110 and asparagine-154 to cysteine. Upon expression of the mutant xylanase, these two cysteine residues will form a disulfide bond. The construction of the plasmid pTrX-DS1 was through ligation of the following overlapping phosphorylated oligonucleotides:

TX-110C	SEQ ID NO:19,
TX-110C-2	SEQ ID NO:20,
TX-103b	SEQ ID NO:21,
XyTv-109	SEQ ID NO:22,
TX-108b	SEQ ID NO:23,
TX-154C	SEQ ID NO:24,
TX-154C-2	SEQ ID NO:25,

into KasI/AvrII-linearized plasmid pTrX in a cassette mutagenesis as shown below.

```
      |                       TX-110C-2
     101 102 103 104 105 106 107 108 109 110 111 112 113 114 115
      G   A   T   K   L   G   E   V   T   C   D   G   S   V   Y
  5'-GC GCC ACA AAA TTA GGC GAA GTC ACT TGT GAT GGA TCC GTA TAT
        3'-G TGT TTT AAT CCG CTT CAG TGA ACA CTA CCT AGG CAT ATA
     KasI|              TX-110C                                 |

                                     |                  TX-103b
 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131
  D   I   Y   R   T   Q   R   V   N   Q   P   S   I   I   G   T
 GAT ATC TAC CGT ACC CAA CGC GTT AAT CAG CCA TCG ATC ATT GGA ACC
 CTA TAG ATG GCA TGG GTT GCG CAA TTA GTC GGT AGC TAG TAA CCT TGG
                      XyTv-109
                          |
 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147
  A   T   F   Y   Q   Y   W   S   V   R   R   N   H   R   S   S
 GCC ACC TTT TAT CAG TAC TGG AGT GTT AGA CGT AAT CAT CGG AGC TCC
 CGG TGG AAA ATA GTC ATG ACC TCA CAA TCT GCA TTA GTA GCC TCG AGG
         |                                  TX-108b
             TX-154C-2
 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163
  G   S   V   N   T   A   C   H   F   N   A   W   A   Q   Q   G
 GGT TCG GTT AAT ACT GCA TGC CAC TTT AAT GCC TGG GCA CAG CAA GGG
 CCA AGC CAA TTA TGA CGT ACG GTG AAA TTA CGG ACC CGT AGT GTT CCC
         |                SphI      TX-154C
         |
 164 165 166 167
  L   T   L   G
 TTA ACC
 AAT TGG GAT C
         AvrII  |
```

C. Construction of the plasmid pTrX-162H-DS1

The mutant TrX-162H-DS1 (SEQ ID NO:56) was identical to TrX-DS1 with a single mutation of glutamine-162 into histidine. The construction of the plasmid pTrX-162D-DS1 was through ligation of oligonucleotides:

TX-162H-3 SEQ ID NO: 26, and

TX-162H-4 SEQ ID NO: 27

into SphI/AvrII-linearized plasmid pTrX-DS1 in a cassette mutagenesis, as shown below.

```
                          TX-162H-3
 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167
  A   C   H   F   N   A   W   A   Q   H   G   L   T   L   G
   5'-C CAC TTC AAT GCA TGG GCA CAG CAC GGG TTA ACC
  GT ACG GTG AAG TTA CGT ACC CGT GTC GTG CCC AAT TGG GAT C-5'
  SphI                                                 AvrII
                          TX-162H-4
```

D. Construction of the plasmid pTrX-162H-DS2

The mutant TrX-162H-DS2 (SEQ ID NO's:57,58) was identical to TrX, but with a covalent disulfide bond between residues-108 and -158, and a mutation glutamine-162 to histidine. The 108/110 disulfide required two single mutations, ie. conversion of both residues valine-108 and alanine-158 to cysteine. Upon expression of the mutant xylanase, these two cysteine residues will form a disulfide bond. The construction of the plasmid pTrX-162H-DS2 was through ligation of the following overlapping phosphorylated oligonucleotides:

TX-108C	SEQ ID NO:45,
TX-108C-2	SEQ ID NO:46,
TX-103b	SEQ ID NO:21,
XyTv-109	SEQ ID NO:22,
TX-108b	SEQ ID NO:23,
TX-158C-162H	SEQ ID NO:47, and
TX-158C-162H-2	SEQ ID NO:48

into the KasI/AvrII-linearized plasmid pTrX in a cassette mutagenesis as shown below.

```
    |                                     TX-108C-2
  101 102 103 104 105 106 107 108 109 110 111 112 113 114 115
    G   A   T   K   L   G   E   C   T   S   D   S   S   V   Y
 5-GC GCC ACA AAA TTA GGC GAA TGC ACT AGT GAT GGA TCC GTA TAT
      3'-G TGT TTT AAT CCG CTT ACG TGA TCA CTA CCT AGG CAT ATA
   KasI|                      TX-108C                         |

                                     |                TX-103b
116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131
  D   I   Y   R   T   Q   R   V   N   Q   P   S   I   I   G   T
GAT ATC TAC CGT ACC CAA CGC GTT AAT CAG CCA TCG ATC ATT GGA ACC
CTA TAG ATG GCA TGG GTT GCG CAA TTA GTC GGT AGC TAG TAA CCT TGG
                          XyTv-109

                         |
132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147
  A   T   F   Y   Q   Y   W   S   V   R   R   N   H   R   S   S
GCC ACC TTT TAT CAG TAC TGG AGT GTT AGA CGT AAT CAT CGG AGC TCC
CGG TGG AAA ATA GTC ATG ACC TCA CAA TCT GCA TTA GTA GCC TCG AGG
        |                                       TX-108b

              TX-158C-162H-2

148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163
  G   S   V   N   T   A   N   H   F   N   C   W   A   Q   H   G
GGT TCG GTT AAT ACT GCA AAT CAC TTT AAT TGC TGG GCA CAG CAC GGG
CCA AGC CAA TTA TGA CGT TTA GTG AAA TTA ACG ACC CGT AGT GTG CCC
         |                        TX-158C-162H

        |
164 165 166 167
  L   T   L   G
TTA ACC
AAT TGG GAT C
          AvrII   |
```

E. Construction of the plasmid pTrX-162H-DS4

The mutant TrX-162H-DS4 (SEQ ID NO's:59, 60) was identical to TrX, but with two covalent disulfide bonds 108/158 and 110/154 and a mutation glutamine-162 to histidine. The two disulfides required four single mutations, ie. conversion of the residues valine-108, serine-110, asparagine-154 and alanine-158 to cysteine. Upon expression of the mutant xylanase, these four cysteine residues will form two disulfide bonds. The construction of the plasmid pTrX-162H-DS4 was through ligation of the following overlapping phosphorylated oligonucleotides:

TX-108C-110C	SEQ ID NO:49,
TX-108C-110C-2	SEQ ID NO:50,
TX-103b	SEQ ID NO:21,
XyTv-109	SEQ ID NO:22,
TX-108b	SEQ ID NO:23,
TX-154C-158C-162H	SEQ ID NO:51 and
TX-154C-158C-162H-2	SEQ ID NO:52

into the KasI/AvrII-linearized plasmid pTrX in a cassette mutagenesis, as shown below.

```
     |                             TX-108C-110C-2
   101 102 103 104 105 106 107 108 109 110 111 112 113 114 115
    G   A   T   K   L   G   E   C   T   C   D   G   S   V   Y
 5'GC GCC ACA AAA TTA GGC GAA TGC ACT TGT GAT GGA TCC GTA TAT
      3'-G TGT TTT AAT CCG CTT ACG TGA ACA CTA CCT AGG CAT ATA
   KasI|                           TX-108C-110C                 |

                                   |                   TX-103b
116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131
 D   I   Y   R   T   Q   R   V   N   Q   P   S   I   I   G   T
GAT ATC TAC CGT ACC CAA CGC GTT AAT CAG CCA TCG ATC ATT GGA ACC
CTA TAG ATG GCA TGG GTT GCG CAA TTA GTC GGT AGC TAG TAA CCT TGG
                       XyTv-109

                        |
132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147
 A   T   F   Y   Q   Y   W   S   V   R   R   N   H   R   S   S
GCC ACC TTT TAT CAG TAC TGG AGT GTT AGA CGT AAT CAT CGG AGC TCC
CGG TGG AAA ATA GTC ATG ACC TCA CAA TCT GCA TTA GTA GCC TCG AGG
        |                                       TX-108b

              TX-154C-158C-162H-2

148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163
 G   S   V   N   T   A   C   H   F   N   C   W   A   Q   H   G
GGT TCG GTT AAT ACT GCA TGC CAC TTT AAT TGC TGG GCA CAG CAC GGG
CCA AGC CAA TTA TGA CGT ACG GTG AAA TTA ACG ACC CGT AGT GTG CCC
         |               SphI           TX-154C-158C-162H

        |
164 165 166 167
 L   T   L   G
TTA ACC
AAT TGG GAT C
        AvrII  |
```

F. Construction of TrX-DS8

The mutant TrX-DS8 was prepared using analogous methods as those outlined above in Sections A to E for the preparation of modified xylanases. TrX-DS8 incorporates the mutations found in N1-TX13 as disclosed in US 5,759,840. This mutations are N10H, Y27M and N29L. In addition, TrX-DS8 includes the following mutations: N44D, Q125A, I129E, Q162H and a disulfide bond between positions 110 and 154. The construction of the plasmid pTrX-DS8 was through ligation of overlapping phosphorylated oligonucleotides as described above.

Trx-DS8 exhibits the property of thermostability (Figure 4a), a pH profile parallelling that of TrX-162-DS1, and greater than 40% of optimum activity at physiological pH, and temperature.

Example 2: Characterization of mutant xylanases

A. Production of xylanases

The culture condition was identical to the well-established protocol described for other *E. coli*-expressed xylanases. A 5 ml of overnight inoculant in 2YT medium (16 g yeast extract, 10 g bacto-tryptone, 5 g NaCl, 1 L of water) containing ampicillin (100 mg/L) was added to 2YT medium (1 L) with ampicillin. The cultures were grown with shaking (200 rpm) at 37°C. After 16 hr. cells were harvested.

B. Purification of different disulfide bond-containing mutant xylanases

Protein samples were prepared from cells by first making an extract of the cells by grinding 10 g of the cell paste with 25 g of alumina powder. After grinding to smooth

mixture, small amounts (5 mL) of ice cold buffer A (10mM sodium acetate, pH 5.5 for BcX mutants) or buffer B (10mM sodium acetate, pH 4.6 for TX mutants) were added and the mixture ground vigorously between additions. The alumina and cell debris were removed by centrifugation of the mixture at 8000 x g for 30 min.

The crude extract was heated at 60°C for 15 min and centrifugation to remove a large amount of precipitate. The supernatant was acidified to pH 4.6, frozen at -20°C overnight, thawed and centrifuged to remove more precipitate.

After the above pretreatment, the cell extract committed to column chromatography and was pumped onto a 50 mL bed volume, S-Sepharose fast flow, cation exchange column (Kabi-Pharmacia, Canada), equilibrated in buffer A. The xylanase was eluted with a 300 mL linear gradient of 0 to 0.3M NaCl in buffer A at a flow rate of 3 mL/min. The xylanase elutes at 100 to 150 mL of the gradient. The fractions are checked on SDS-PAGE, and those fractions having most of the xylanase were pooled, and concentrated by ultrafiltration using 3000 dalton molecular weight cutoff membranes (Amicon YM3). The concentrated material (5 mL) was then applied to a 1.5 cm x 85 cm TSK-HW50S gel filtration column, equilibrated in 50 mM ammonium acetate pH 6. The xylanase eluted at a volume of 90 to 100 mL. These fractions were analyzed by SDS-PAGE, and the peaks pooled as pure xylanase. The protein was quantified using the extinction co-efficient at 280 nm.

C. Standard assay for the measurement of enzymatic activity

The quantitative assay determined the number of reducing sugar ends generated from soluble xylan. The substrate for this assay was the fraction of birchwood xylan which dissolved in water from a 5% suspension of birchwood xylan (Sigma Chemical Co.). After removing the insoluble fraction, the supernatant was freeze dried and stored in a desiccator. The measurement of specific activity was performed as follows. Reaction mixtures containing 100 L of 30 mg/mL xylan previously diluted in assay buffer (50 mM sodium citrate, pH 5.5

or the pH optimum of the tested xylanase), 150 L assay buffer, 50 L of enzyme diluted in assay buffer were incubated at 40°C. At various time intervals 50 L portions were removed and the reaction stopped by diluting in 1 mL of 5mM NaOH. The amount of reducing sugars was determined with the hydroxybenzoic acid hydrazide reagent (HBAH) (Lever, 1972, Analytical Biochem 47:273-279). A unit of enzyme activity was defined as that amount generating 1 mol reducing sugar in 1 minute at 40°C.

For the comparison between mutant and the wild type xylanases (TABLE 3), the specific activities of a xylanase was converted to the relative activity which is its calculated in percentage as compared to the specific activity of the natural xylanase.

TABLE 3. Relative activity of TrX xylanases

Xylanase	Relative activity %
natl. TrX	100*
TrX	103
TrX-DS1	116
TrX-162H-DS1	102
TrX-162H-DS4	91

* The specific activity of the natural TrX (770 U/mg) was normalized to 100%.

As can be seen form Table 3, the specific enzymatic activities of the mutant xylanases at 40°C have not been changed significantly as compared to the natural xylanases.

Example 3: Thermostability of mutant xylanases

This was a test of the tolerance of xylanase to incubation at a set temperature, without any substrate. The xylanase (150 g/mL) in assay buffer (50 mM sodium citrate) was

incubated at a set temperature or set period of time. Aliquots were cooled to room temperature (around 20°C), the residual enzymatic activity of all samples was determined via the HBAH assay at 40°C, as stated in Example 2C.

(A) Effect of length of incubation

The effect of the length of incubation on the activity of xylanase samples was determined at 62.5°C at pH 5.5 (Figure 3). Aliquots were removed at 0, 5, 10, 20, 30, 40 and 60 min for the determination of residual activity. The residual enzymatic activity at 0 min was normalized to 100%.

After 5 mins of incubation, the wild type TrX and the Q162H mutant TrX-162H (US 5,759,840) almost lost all residual activity, while the mutant TrX-DS1 with a disulfide bond, retained 60% of it residual activity. However, it retained only 20% of its activity at 20 mins and lost all activity at 40 min. In contrast, the mutant TrX-162H-DS1, with the additional mutation of Q162H, showed superior thermostability by retaining about 87% of its activity at 20 min, 78% at 40 min and 68% at 60 min. The mutant TrX-162H-DS4 with both 108/158 and 110/154 disulfide bonds retained 84% activity after 60 min..

(B) Effect of incubation temperatures on the residual activity of mutant TrX.

Thermostability of mutant TrX enzymes was also determined by tolerance of different incubation temperatures. Samples of xylanases were incubated in 50 mM sodium citrate buffer (pH 5.5) at different temperatures (48, 52, 56, 60, 64, 68, 70 and 72°C) for 30 min. The residual enzymatic activity of the samples was determined, with the residual activity at 48°C normalized to 100% (see Figures 4(a) and 4(b)). The T_{50}, which is the incubation temperature allowing the maintenance of 50% residual activity after 30 min, was determined for each mutant TrX.

Without wishing to be bound by theory, the higher T_{50} of TrX-162H-DS1 (65°C) versus TrX-DS1 (61°C) demonstrates the enhancement of thermostability by the mutation

Q162H in the disulfide mutants. The double disulfide mutant TrX-162H-DS4 also exhibited high stability with a T_{50} gain of +14°C over the natural TrX. Comparison of T_{50} of TrX-162H-DS1 (65°C) and TrX-162H-DS2 (59°C) indicates that the 110/154 disulfide in TrX-162H-DS1 contributes greater thermostability than the108/158 dislufide in the latter. TrX-DS8 also exhibited high thermostability, with a T_{50} gain of +16°C when compared to natural TrX.

(C) Effective incubation temperature

In the following example, a model study of the effect of the enzyme formulation on thermostability of the combination mutant was conducted in the presence of an additive, glycerol. The unmodified TrX and the mutant TrX xylanases were incubated for 30 min at 20, 50, 60, 70, 80 and 90°C in a buffer (pH 5.0) with 40% glycerol. The residual activity was determined by the HBAH assay. The residual enzymatic activity at 0 min was normalized to 100% (Figure 5).

At 50°C, all TrX samples retained their enzymatic activity. At 60°C, the wild type TrX retained 75% of its activity while TrX-DS1 and TrX-162H-DS1 retained 80 and 100% respectively (Figure 5). At 70°C, TrX-DS1 and TrX-162H-DS1 maintained 10 and 98% respectively. At 90 min, the latter retained 65% of the residual activity.

(D) Effect of incubation time on the residual activity of TrX-162H-DS1 at 90°C

Sample of TrX-162H-DS1 in 40% glycerol and buffer were incubated in a covered circulating water bath (Haake type F 4391, with a fluctuation of 0.1°C) at 90°C. Temperature of the water bath was confirmed with a thermocouple. Aliqiots were removed at 0, 5, 10 and 30 min for assay of residual activity. The residual enzymatic activity at 0 min was normalized to 100%.

At 5, 10 and 30 min, TrX-162H-DS1 retained 90, 85 and 65% of the residual activity respectively (Figure 6).

Example 4: Temperature/activity profile of mutant xylanases

This was a test on the effect on different temperatures to the enzymatic activity of the xylanase in the hydrolysis of soluble xylan. The procedure was identical to the standard assay (Example 2 C) with changes in the incubation temperature and time. The enzymes (1.5 μg/mL) and soluble xylanase in 50 mM sodium citrate buffer of pH 4.5 were mixed and incubated in a circulating water bath at different temperatures. After 30 min, the amount of reducing sugars released from xylan was determined by HBAH and was calculated as relative activity, with the value at temperature optimum as 100%.

The effect of temperature on the hydrolysis of xylan was shown in Figure 7. The natural TrX, TrX-DS1, TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4 enzymes all had the same temperature/activity profile, and the only difference is in the greater activity (80%) in mutant TrX-162H-DS4 as compared to the others (45%) at 60°C. These results indicate that the disulfide mutation, along with the Q162H mutation, has little or no effect on the optimal temperature (50°C) of TrX. In addition, all of the enzymes shown in the figure exhibit at least 40% of their optimum activity from about 40 to about 50°C, which is suitable for feed pelleting applications.

Example 5: pH/activity profile of mutant xylanases

This was a test of the effect of different pH on the enzymatic activity of the xylanase in the hydrolysis of soluble xylan at the approximate physiological temperature of digesta.

The procedure was identical to the standard assay (Example 2 C) with changes in the incubation temperature and time. The *Trichoderma* enzymes natural TrX and mutant TrX (30 μg/mL) and soluble xylan in 50 mM sodium citrate buffers of pH 3-8 were incubated together at 40°C for 7 min. The amount of reducing sugars released from xylan was determined by HBAH and was calculated as relative activity, with the value at pH optimum as 100%.

The profile of the effect of pH on the enzymatic activity of TrX, TrX-162H-DS1 and TrX-162H-DS2 (Figure 8) are similar, thus indicating little or no effect of the mutations (disulfide bond formation and Q162H) on the pH optimum. The pH profile for TrX-DS8 was also similar to these modified xylanases (data not shown). All of the enzymes shown in the figure exhibit at least 40% of their optimum activity from about pH 3.5 to about pH 6, which is suitable for feed pelleting applications.

The double disulfide mutant TrX-162H-DS4 differed by showing slightly greater activity at the pH range higher than 6. At the acidic pH of 4-6 TrX, TrX-162H-DS1, TrX-162H-DS2 and TrX-162H-DS4 maintained at least 75% optimal activity.

All citations listed herein are incorporated by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

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CLAIMS

1. An isolated, modified, Family 11 xylanase characterized in exhibiting at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 50°C, said xylanase being thermostable.

2. The isolated xylanase of claim 1 wherein said xylanase is characterized in exhibiting at least 40% of optimal activity from about 40 to about 60°C.

3. The isolated xylanase of claim 2 wherein said thermostability is characterized by said xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 70°C in the presence of 40% glycerol.

4. The isolated xylanase of claim 2 wherein said thermostability is characterized by said xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 80°C in the presence of 40% glycerol.

5. The isolated xylanase of claim 2 wherein said thermostability is characterized by said xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 30 minutes at 90°C in the presence of 40% glycerol.

6. The isolated xylanase of claim 2 wherein said thermostability is characterized by said xylanase exhibiting at least 30% of optimal activity after a pre-incubation step for 60 minutes at 62.5°C.

7. The isolated xylanase of claim 5 wherein said thermostability is determined in the absence of stabilizer.

8. The isolated xylanase of claim 6 wherein said thermostability is determined in the absence of stabilizer.

9. The isolated xylanase of claim 2, wherein said xylanase is a modified xylanase.

10. The isolated xylanase of claim 9, wherein said xylanase is a Family 11 xylanase.

11. The isolated xylanase of claim 10, wherein said Family 11 xylanase is a *Trichoderma* xylanase.

12. A modified xylanase comprising a basic amino acid at position 162 (TrX numbering) or its equivalent, exhibiting at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 60°C, said modified xylanase being thermostable.

13. The modified xylanase of claim 12, wherein said basic amino acid is selected from the group consisting of lysine, arginine and histidine.

14. The modified xylanase of claim 13, wherein said basic amino acid is histidine.

15. The modified xylanase of claim 9 comprising at least one disulfide bridge.

16 The modified xylanase of claim 9 comprising two disulfide bridges.

17. The modified xylanase of claim 9 comprising a basic amino acid at position 162 (TrX numbering) or its equivalent position, and at least one disulfide bridge.

18 The modified xylanase of claim 9 selected from the group consisting of TrX-162H-DS1, TrX-162H-DS2, TrX-162H-DS4, and TrX-DS8.

19. The modified xylanase of claim 18, wherein said xylanase is TrX-162H-DS1.

20. The modified xylanase of claim 18, wherein said xylanase is TrX-162H-DS2.

21. The modified xylanase of claim 18, wherein said xylanase is TrX-162H-DS4.

22. The modified xylanase of claim 18, wherein said xylanase is TrX-DS8.

23. A method of obtaining a Family 11 xylanase comprising:
   i) selecting an organism that expresses xylanase activity, and obtaining said xylanase from said organism;
   ii) determining whether said xylanase exhibits at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 60°C; and
   iii) determining whether said xylanase is thermostable, and whether said xylanase is a Family 11 xylanase; and
   iv) retaining said xylanase that express these properties.

24. The method of claim 24, wherein step i) includes partially purifying said xylanase.

25. A method of preparing animal feed comprising applying the isolated xylanase of claim 1 onto said animal feed to produce a xylanase-animal feed combination, and heat sterilizing said xylanase-animal feed combination.

26. The method of claim 25, wherein said animal feed is a poultry or swine feed.

27. A method of preparing animal feed comprising, applying the xylanase obtained from step iv) of claim 23 onto said animal feed to produce a xylanase-animal feed combination, and heat sterilizing said xylanase-animal feed combination.

28. The method of claim 27, wherein said animal feed is a poultry or swine feed.

29. An isolated recombinant xylanase characterized in exhibiting at least 40% of optimal activity from about pH 3.5 to about pH 6.0, and from about 40 to about 50°C, said recombinant xylanase being thermostable.

30. The modified xylanase of claim 12 comprising at least one disulfide bridge.

31. The modified xylanase of claim 12 comprising two disulfide bridges.

32. The isolated, modified, xylanase of claim 1, said xylanase obtained from an organisms selected from the group consisting of *Aspergillus niger*, *Aspergillus kawachii*, *Aspergillus tubigensis*, *Bacillus circulans*, *Bacillus pumilus*, *Bacillus subtilis*, *Cellulomonas fimi*, *Chainia spp.*, *Clostridium acetobutylicum*, *Clostridium stercorarium*, *Fibrobacter succinognees*, *Neocallimasterix patriciarum*, , *Nocardiopsis dassonvillei*, *Ruminococcus flavefaciens*, *Schizophyllum commune*, *Streptomyces lividans*, *Streptomyces lividans*, *Streptomyces sp. No. 36a*, *Streptomyces thermoviolaceus*, *Thermomonospora fusca*, , *Trichoderma harzianum*, *Trichoderma reesei*, *Trichoderma reesei*, and*Trichoderma viride*.

```
Ca    23                                                    S AFNTQAAP   31
Cs     1                                                             G    1

Bp     1  RTITNNEMGN  HSGYDYELWK DYGNT-SMTL NNGGAFSAGW N--NIGNA  45
Ca    32  KTITSNEIGV  NGGYDYELWK DYGNT-SMTL KNGGAFSCQW S--NIGNA  76
Cs     2  RIIYDNETGT  HGGYDYELWK DYGNT-IMEL NDGGTFSCQW S--NIGNA  46
Rf     1  SAADQQTRGN  VGGYDYEMWN QNGQGQASMN PGAGSFTCSW S--NIENF  46
Tr2    1   QTIQPGTGY  NNGYFYSYWN DGHGGVTYTN GPGGQFSVNW S--NSGNF  45
Tv     1   QTIQPGTGF  NNGYFYSYWN DGHGGVTYTN GPGGQFSVNW S--NSGNF  45
Th     1   QTIGPGTGY  SNGYYYSYWN DGHAGVTYTN GGGGSFTVNW S--NSGNF  45
Sc     1   SGTPSSTGT  DGGYYYSWWT DGAGDATYQN NGGGSYTLTW SG-NNGNL  46
An     1           S  AGINYVQNYN GNLGDFTY-D ESAGTFSMYW EDGVSSDF  38
AT     1           S  AGINYVQNYN QNLGDFTY-D ESAGTFSMYW EDGVSSDF  38
Tr1    1              ASINYDQNYQ TGG-QVSYS- PSNTGFSVNW N--TQDDF  34
Ss     1   ATTIT-NETGY D-GMYYSFWT DGGGSVSMTL NGGGSYSTRW T--NCGNF  45
SlB    1   DTVVTTNQEGT NNGYYYSFWT DSQGTVSMNM GSGGQYSTSW R--NTGNF  47
SlC    1   ATTITTNQTGT D-GMYYSFWT DGGGSVSMTL NGGGSYSTQW T--NCGNF  46
Tf     1   AVTSNETGY  HDGYFYSFWT DAPGTVSMEL GPGGNYSTSW R--NTGNF  45
Bc     1              ASTDYWQNWT DGGGIVNAVN GSGGNYSVNW S--NTGNF  36
Bs     1              ASTDYWQNWT DGGGIVNAVN GSGGNYSVNW S--NTGNF  36


Bp    46  LFRK-GKKFD ST-RTHHQLG NISINYNASF N-PSGNSYLC VYGWTQSP   90
Ca    77  LFRK-GKKFN DT-QTYKQLG NISVNYNCNY Q-PYGNSYLC VYGWTSSP  121
Cs    47  LFRK-GRKFN SD-KTYQELG DIVVEYGCDY N-PNGNSYLC VYGWTRNF   91
Rf    47  LARM-GKNYD SQKKNYKAFG NIVLTYDVEY T-PRGNSYMC VYGWTRNP   92
Tr2   46  VGGK-GWQPG TKNKV----- ---INFS-GS YNPNGNSYLS VYGWSRNP   83
Tv    46  VGGK-GWQPG TKNKV----- ---INFS-GS YNPNGNSYLS VYGWSRNP   83
Th    46  VGGK-GWQPG TKNKV----- ---INFS-GS YNPNGNSYLS IYGWSRNP   83
Sc    47  VGGK-GWNPG AASRS----- ---ISYS-GT YQPNGNSYLS VYGWTRSS   84
An    39  VVGL-GWTTG SSNA------ ---ITYSAEY SASGSSSYLA VYGWVNYP   76
At    39  VVGLGGWTTG SSNA------ ---ITYSAEY SASGSASYLA VYGWVNYP   77
Tr1   35  VVGV-GWTTG SSAP------ ---INFGGSF SVNSGTGLLS VYGWSTNP   72
Ss    46  VAGK-GWANG GR-RT----- ---VRYT-GW FNPSGNGYGC LYGWTSNP   82
SlB   48  VAGK-GWANG GR-RT----- ---VQYS-GS FNPSGNAYLA LYGWTSNP   84
SlC   47  VAGK-GWSTG DGN------- ---VRYN-GY FNPVGNGYGC LYGWTSNP   82
Tf    46  VAGK-GWATG GR-RT----- ---VTYS-AS FNPSGNAYLT LYGWTRNP   82
Bc    37  VVGK-GWTTG SPFRT----- ---INYNAGV WAPNGNGYLT LYGWTRSP   75
Bs    37  VVGK-GWTTG SPFRT----- ---INYNAGV WAPNGNGYLT LYGWTRSP   75
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FIGURE 1

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Bp    91  LAEYYIVDSW GTYR-PT--G AYKGSFYADG GTYDIYETTR VNQPSIIG 135
Ca   122  LVEYYVIDSW GSWRPP--GG TSKGTITVDG GIYDIYETTR INQPSIQG 167
Cs    92  LVEYYIVESW GSWRPP--GA TPKGTITQWMAGTYEIYETTR VNQPSIDG 138
Rf    93  LMEYYIVEGW GDWRPPGNDG EVKGTVSANG NTYDIRKTMR YNQPSLDG 140
Tr2   84  LIEYYIVENF GTYN-PSTGA TKLGEVTSDG SVYDIYRTQR VNQPSIIG 130
Tv    84  LIEYYIVENF GTYN-PSTGA TKLGEVTSDG SVYDIYRTQR VNQPSIIG 130
Th    84  LIEYYIVENF GTYN-PSTGA TKLGEVTSDG SVYDIYRTQR VNQPSIIG 130
Sc    85  LIEYYIVESY GSYD-PSSAA SHKGSVTCNG ATYDILSTWR YNAPSIDG 131
An    77  GAEYYIVEDY GDYN-PCSSA TSLGTVYSDG STYQVCTDTR INEPSITG 123
At    78  QAEYYIVEDY GDYN-PCSSA TSLGTVYSDG STYQVCTDTR INEPSITG 124
Tr1   73  LVEYYIMEDN HNY--PAQ-G TVKGTVTSDG ATYTIWENTR VNEPSIQG 117
Ss    83  LVEYYIVDNW GSYR-PT--G ETRGTVHSDG GTYDIYKTTR YNAPSVEA 127
SlB   85  LVEYYIVDNW GTYR-PT--G EYKGTVTSDG GTYDIYKTTR VNKPSVEG 129
SlC   83  LVEYYIVDNW GSYR-PT--G TYKGTVSSDG GTYDIYQTTR YNAPSVEG 127
Tf    83  LVEYYIVESW GTYR-PT--G TYMGTVTTDG GTYDIYKTTR YNAPSIEG 127
Bc    76  LIEYYVVDSW GTYR-PT--G TYKGTVKSDG GTYDIYTTTR YNAPSIDG 120
Bs    76  LIEYYVVDSW GTYR-PT--G TYKGTVKSDG GTYDIYTTTR YNAPSIDG 120
```

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Bp   136  -IATFKQYWS VRQTKRTS-- ------GTVS VSAHFRKWES LGMPM-GK 174
Ca   168  -NTTFKQYWS VRRTKRTS-- ------GTIS VSKHFAAWES KGMPL-GK 206
Cs   139  -TATFQQYWS VRTSKRTS-- ------GTIS VTEHFKQWER MGMRM-GK 177
Rf   141  -TATFPQYWS VRQTSGSANN QTNYMKGTID VSKHFDAWSA AGLDMSGT 187
Tr2  131  -TATFYQYWS VRRNHR-S-S ------GSVN TANHFNAWAQ QGLTL-GT 168
Tv   131  -TATFYQYWS VRRTHR-S-S ------GSVN TANHFNAWAQ QGLTL-GT 168
Th   131  -TATFYQYWS VRRNHR-S-S ------GSVN TANHFNAWAS HGLTL-GT 168
Sc   132  -TQTFEQFWS VRNPKKAPGG SIS---GTVD VQCHFDAWKG LGMNLGSE 175
An   124  -TSTFTQYFS VRESTRTS-- ------GTVT VANHFNFWAQ HGFGN-SD 162
At   125  -TSTFTQYFS VRESTRTS-- ------GTVT VANHFNFWAH HGFHN-SD 163
Tr1  118  -TATFNQYIS VRNSPR-T-S ------GTVT VQNHFN-WAS LGLHLGQM 155
Ss   128  -PAAFDQYWS VRQSKVT--S ------GTIT TGNHFDAWAR AGMNMGNF 168
SlB  130  TR-TFDQYWS VRQSKR-TG- ------GTIT TGNHFDAWAR AGMPLGNF 168
SlC  128  TK-TFQQYWS VRQSKVTSGS ------GTIT TGNHFDAWAR AGMNMGQF 168
Tf   128  TR-TFDQYWS VRQSKRTS-- ------GTIT AGNHFDAWAR HGMHLGTH 166
Bc   121  DRTTFTQYWS VRQSKRPTGS N-----ATIT FTNHVNAWKS HGMNLGSN 163
Bs   121  DRTTFTQYWS VRQSKRPTGS N-----ATIT FSNHVNAWKS HGMNLGSN 163
```

FIGURE 1 (CONT'D)

3/12

Bp	174	MYETAFTVEG	YQSSGSANVM	TNQLFIGN		201
Ca	207	MHETAFNIEG	YQSSGKADVN	SMSINIGK		233
Cs	178	MYEVALTVEG	YQSSGYANVY	KNEIRIGANP....		
Rf	188	LYEVSLNIEG	YRSNGSANVK	SVSV		211
Tr2	169	MDYQIVAVEG	YFSSGSASI-	TVS		190
Tv	169	MDYQIVAVEG	YFSSGSASI-	TVS		190
Th	169	MDYQIVAVEG	YFSSGSASI-	TVS		190
Sc	176	HNYQIVATEG	YQSSGTATI-	TVT		197
An	163	FNYQVMAVEA	WSGAGSASV-	TISS		184
At	164	FNYQVVAVEA	WSGAGSAAV-	TISS		185
Tr1	156	MNYQVVAVEG	WGGSGSASQ-	SVSN		178
Ss	167	RYYMINATEG	YQSSGSSTI-	TVSG		189
SlB	169	SYYMINATEG	YQSSGTSSI-	NVGG..........		
SlC	169	RYYMINATEG	YQSSGSSNI-	TVSG		191
Tf	167	D-YMIMATEG	YQSSGSSNVT	LGTS..........		
Bc	164	WAYQVMATEG	YQSSGSSNV-	TVW		185
Bs	164	WAYQVMATEG	YQSSGSSNV-	TVW		185

Bp	*Bacillus pumilus*
Ca	*Clostridium acetobutylicum* P262 XynB
Cs	*Clostridium stercorarium* xynA
Rf	*Ruminococcus flavefaciens*
Tr2	*Trichoderma reesei* XYN II
Tv	*Trichoderma viride*
Th	*Trichoderma harzianum*
Sc	*Schizophyllum commune* Xylanase A
An	*Aspergillus niger*, var. *awamori*
At	*Aspergillus tubigensis*
Tr1	*Trichoderma reesei* XYN I
Ss	*Streptomyces* sp. 36a
SlB	*Streptomyces lividans* Xln B
SlC	*Streptomyces lividans* Xln C
Tf	*Thermomonospora fusca* TfxA
Bc	*Bacillus circulans*
Bs	*Bacillus subtilis*

FIGURE 1 (CONT'D)

```
                                        |                        st
                                  5'-CT AGC TAA GGA GG CTG CAG ATG
                                            G ATT CCT CC GAC GTC TAC
                                       NheI |           PstI

                                       TrX-1
  1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16
  Q   T   I   Q   P   G   T   G   Y   N   N   G   Y   F   Y   S
CAA ACA ATA CAA CCA GGA ACC GGT TAC AAC AAC GGT TAC TTT TAC AGC
GTT TGT TAT GTT GGT CCT TGG CCA ATG TTG TTG CCA ATG AAA ATG TCG
        TrX-8               AgeI                |

        |                   XyTv-2
 17  18  19  20  21  22  23  24  25  26  27  28  29  30  31  32
  Y   W   N   D   G   H   G   G   V   T   Y   T   N   G   P   G
TAT TGG AAC GAT GGC CAT GGT GGT GTT ACC TAT ACA AAC GGG CCC GGA
ATA ACC TTG CTA CCG GTA CCA CCA CAA TGG ATA TGT TTG CCC GGG CCT
                      NcoI                  XyTv-7      ApaI

                                                |
 33  34  35  36  37  38  39  40  41  42  43  44  45  46  47  48
  G   Q   F   S   V   N   W   S   N   S   G   N   F   V   G   G
GGC CAA TTT AGC GTC AAT TGG TCT AAC TCC GGA AAC TTC GTA GGT GGA
CCG GTT AAA TCG CAG TTA ACC AGA TTG AGG CCT TTG AAG CAT CCA CCT
                      MunI  |         BspEI

                          TrX-3
 49  50  51  52  53  54  55  56  57  58  59  60  61  62  63  64
  K   G   W   Q   P   G   T   K   N   K   V   I   N   F   S   G
AAA GGT TGG CAA CCC GGG ACC AAA AAT AAG GTG ATC AAC TTC TCT GGA
TTT CCA ACC GTT GGG CCC TGG TTT TTA TTC CAC TAG TTG AAG AGA CCT
                  XmaI                  TrX-6

                          |
 65  66  67  68  69  70  71  72  73  74  75  76  77  78  79  80
  S   Y   N   P   N   G   N   S   Y   L   S   V   Y   G   W   S
TCT TAT AAT CCG AAT GGG AAT TCA TAC TTA AGC GTC TAT GGC TGG TCT
AGA ATA TTA GGC TTA CCC TTA AGT ATG AAT TCG CAG ATA CCG ACC AGA
  |                      EcoRI       AflII

   XyTv-4                                                    |
 81  82  83  84  85  86  87  88  89  90  91  92  93  94  95
  R   N   P   L   I   E   Y   Y   I   V   E   N   F   G   T
AGA AAC CCA CTG ATT GAA TAT TAC ATT GTC GAA AAT TTC GGT AC
TCT TTG GGT GAC TAA CTT ATA ATG TAA CAG CTT TTA AAG C
Xba I               XyTv-5                            | KpnI
```

FIG. 2

```
                               XyTv-101
         92  93  94  95  96  97  98  99 100 101 102 103 104 105
 V   D   N   F   G   T   Y   N   P   S   T   G   A   T   K   L
TC  GAC AAT TTC GGT ACC TAC AAT CCG AGT ACC GGC GCC ACA AAA TTA
      G TTA AAG CCA TGG ATG TTA GGC TCA TGG CCG CGG TGT TTT AAT
SalI            KpnI            XyTv-110    KasI/NarI

                                                  XyTv-102
106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121
 G   E   V   T   S   D   G   S   V   Y   D   I   Y   R   T   Q
GGC GAA GTC ACT AGT GAT GGA TCC GTA TAT GAT ATC TAC CGT ACC CAA
CCG CTT CAG TGA TCA CTA CCT AGG CAT ATA CTA TAG ATG GCA TGG GTT
              SpeI        BamHI                      XyTv-109

                                              TrX-103
122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137
 R   V   N   Q   P   S   I   I   G   T   A   T   F   Y   Q   Y
CGC GTT AAT CAG CCA TCG ATC ATT GGA ACC GCC ACC TTT TAT CAG TAC
GCG CAA TTA GTC GGT AGC TAG TAA CCT TGG CGG TGG AAA ATA GTC ATG
MluI                ClaI

138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153
 W   S   V   R   R   N   H   R   S   S   G   S   V   N   T   A
TGG AGT GTT AGA CGT AAT CAT CGG AGC TCC GGT TCG GTT AAT ACT GCG
ACC TCA CAA TCT GCA TTA GTA GCC TCG AGG CCA AGC CAA TTA TGA CGC
    TrX-108                      SacI

                        XyTv-104
154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169
 N   H   F   N   A   W   A   Q   Q   G   L   T   L   G   T   M
AAT CAC TTT AAT GCA TGG GCA CAG CAA GGG TTA ACC CTA GGT ACA ATG
TTA GTG AAA TTA CGT ACC CGT GTC GTT CCC AAT TGG GAT CCA TGT TAC
             NsiI         XyTv-107                AvrII

                             XyTv-105
170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185
 D   Y   Q   I   V   A   V   E   G   Y   F   S   S   G   S   A
GAT TAT CAA ATC GTA GCG GTG GAA GGC TAC TTC TCG AGT GGT TCC GCT
CTA ATA GTT TAG CAT CGC CAC CTT CCG ATG AAG AGC TCA CCA AGG CGA
                             XyTv-106          XhoI

186 187 188 189 190
 S   I   T   V   S
AGT ATT ACA GTG AGC TAA A
TCA TAA TGT CAC TCG ATT TCT AG
                        BglII
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FIG. 2 (CONT'D)

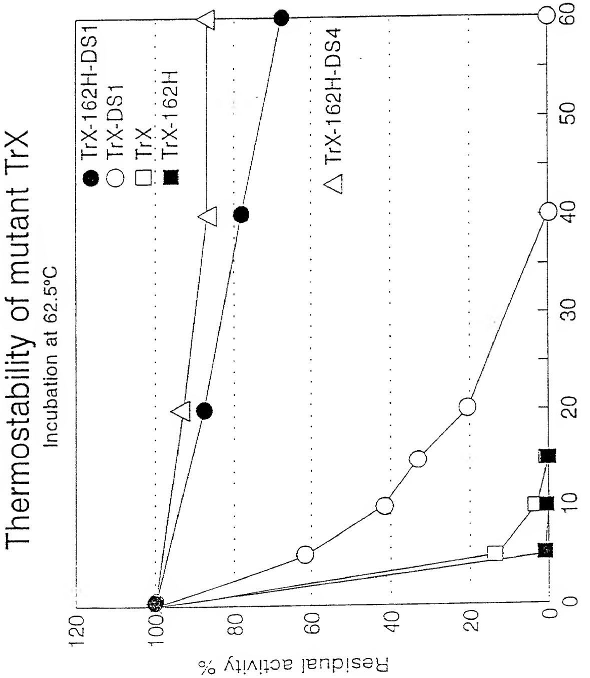
Thermostability of mutant TrX
Incubation at 62.5°C
TrX-162H-DS1
TrX-DS1
TrX
TrX-162H
TrX-162H-DS4
Residual activity %
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Incubation time (min)

FIG. 3

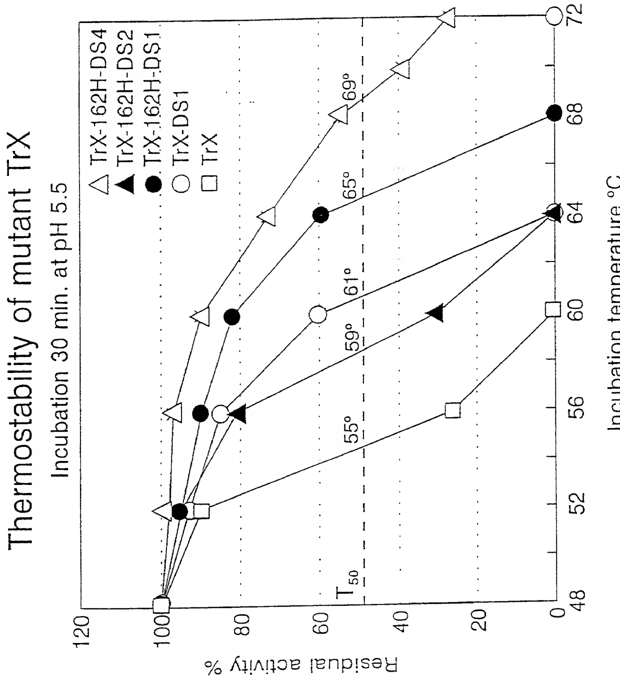
Thermostability of mutant TrX
Incubation 30 min. at pH 5.5
TrX-162H-DS4
TrX-162H-DS2
TrX-162H-DS1
TrX-DS1
TrX
Residual activity %
Incubation temperature °C
T_{50}
55°
59°
61°
65°
69°
120
100
80
60
40
20
0
48
52
56
60
64
68
72

FIG. 4A

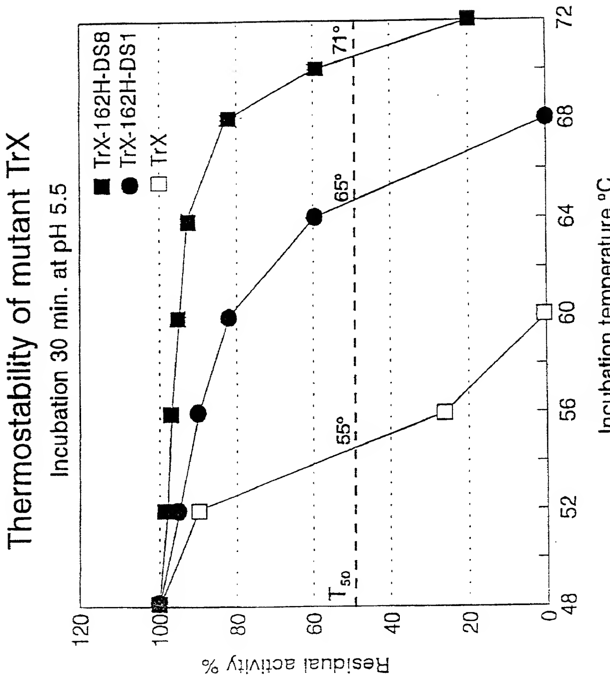
Thermostability of mutant TrX
Incubation 30 min. at pH 5.5
TrX-162H-DS8
TrX-162H-DS1
TrX
T_{50}
55°
65°
71°
Residual activity %
120
100
80
60
40
20
0
48
52
56
60
64
68
72
Incubation temperature °C

FIG. 4B

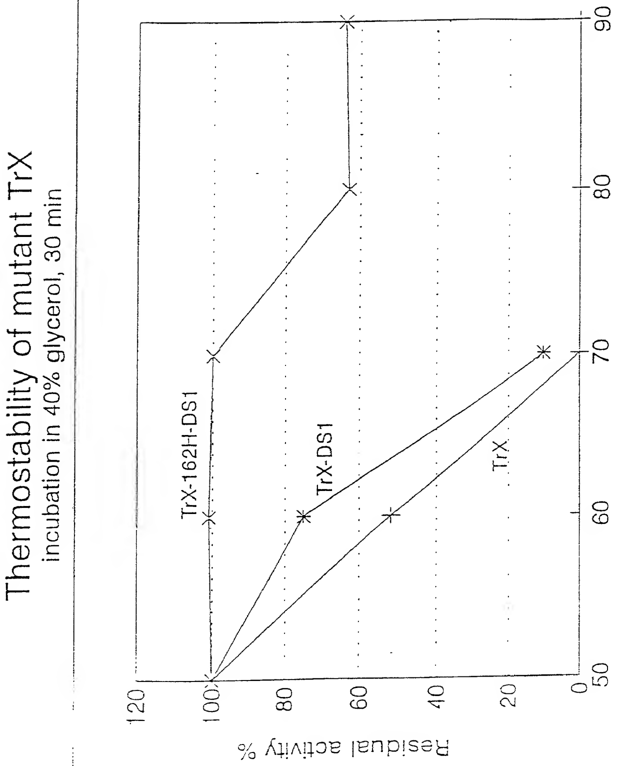
Thermostability of mutant TrX
incubation in 40% glycerol, 30 min
TrX-162H-DS1
TrX-DS1
TrX
Residual activity %
120
100
80
60
40
20
0
50
60
70
80
90
Incubation temperature °C

FIG. 5

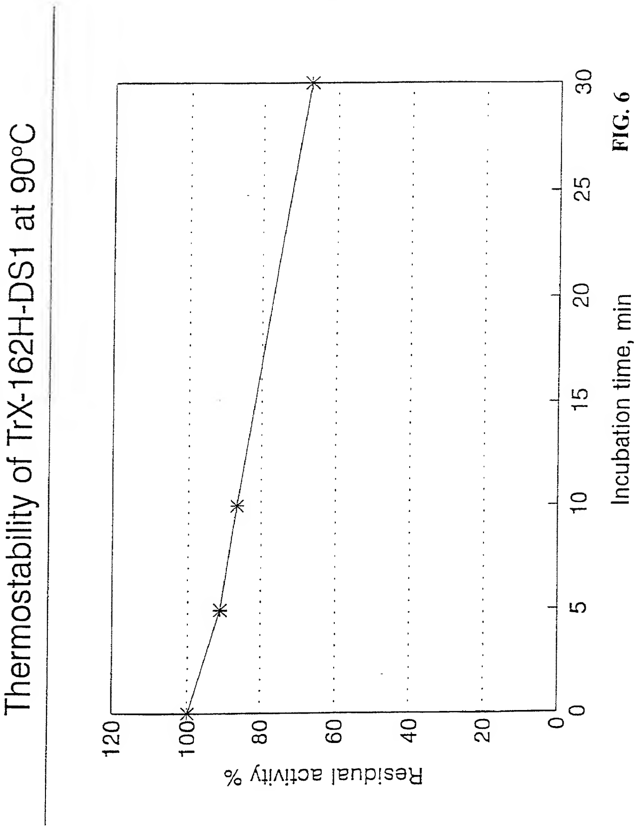
Thermostability of TrX-162H-DS1 at 90°C
Residual activity %
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Incubation time, min

FIG. 6

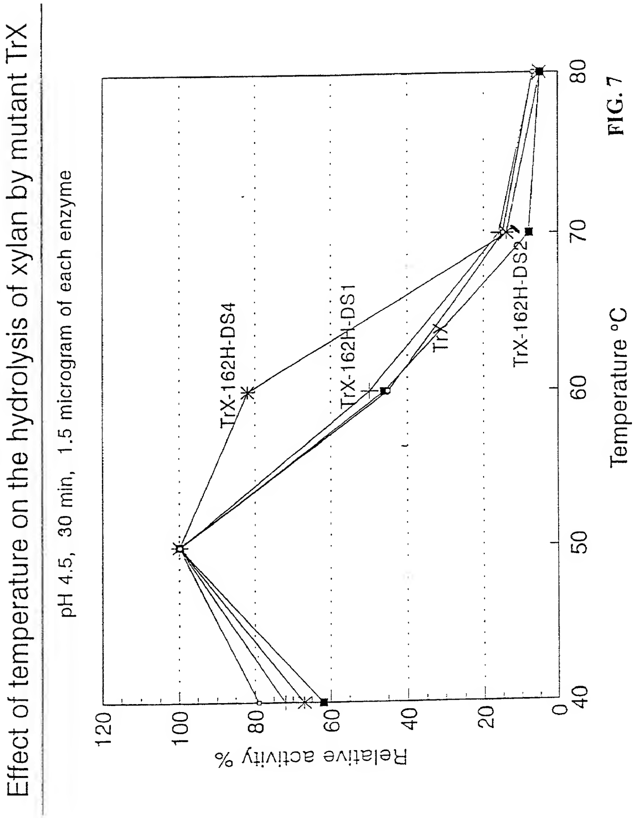
Effect of temperature on the hydrolysis of xylan by mutant TrX
pH 4.5, 30 min, 1.5 microgram of each enzyme
Relative activity %
120
100
80
60
40
20
0
40
50
60
70
80
Temperature °C
TrX-162H-DS4
TrX-162H-DS1
TrX
TrX-162H-DS2

FIG. 7

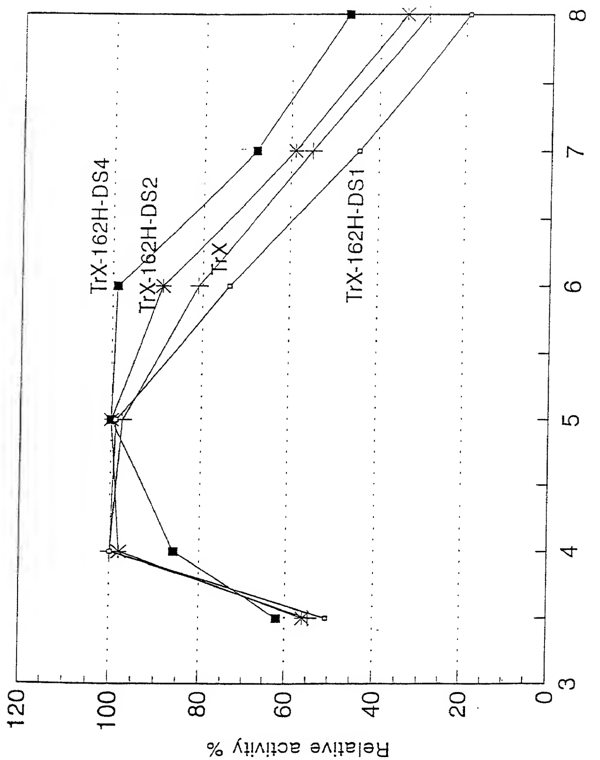
TrX-162H-DS4
TrX-162H-DS2
TrX
TrX-162H-DS1
Relative activity %
pH
120
100
80
60
40
20
0
3
4
5
6
7
8

FIG. 8

#4

ATTORNEY DOCKET NO. 07121.0001U1

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(X) Original ( ) Supplemental ( ) Substitute ( ) PCT

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **"THERMOSTABLE XYLANASES,"** which is described and claimed in the specification

(check one) [ ] which is attached hereto, or
[X] which was filed on 16 May 2001, as United States Application No. 09/856,025 and with amendments through (if applicable), or
[ ] in International Application No., filed on , and as amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known by me to be material to the patentability of the claims of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119 (a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) or §365(b) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATIONS: (ENTER BELOW IF APPLICABLE)			PRIORITY CLAIMED (MARK APPROPRIATE BOX BELOW)	
APP. NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	YES	NO
PCT/CA99/01093	PCT	16 November 1999	X	

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application listed below.

APPLICATION NUMBER	FILING DATE
60/108,504	16 November 1998

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information known by me to be material to the patentability of the claims of this application as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS (MARK APPROPRIATE COLUMN BELOW)		
		PATENTED	PENDING	ABANDONED

I hereby appoint the following attorneys and/or agent(s) to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith:

23859
PATENT TRADEMARK OFFICE

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Atlanta, Georgia 30303-1811

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment,

or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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SEQUENCE LISTING

<110> Wing Dr., Sung L.
Tolan Dr., Jeffrey S.

<120> Xylanases with Improved Performance in Feed Pelleting Applications

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             20                  25                  30

Gly Val Ser Ser Asp Phe Val Val Gly Leu Gly Trp Thr Thr Gly Ser
         35                  40                  45

Ser Asn Ala Ile Thr Tyr Ser Ala Glu Tyr Ser Ala Ser Gly Ser Ser
     50                  55                  60

Ser Tyr Leu Ala Val Tyr Gly Trp Val Asn Tyr Pro Gly Ala Glu Tyr
 65                  70                  75                  80

Tyr Ile Val Glu Asp Tyr Gly Asp Tyr Asn Pro Cys Ser Ser Ala Thr
                 85                  90                  95

Ser Leu Gly Thr Val Tyr Ser Asp Gly Ser Thr Tyr Gln Val Cys Thr
            100                 105                 110

Asp Thr Arg Ile Asn Glu Pro Ser Ile Thr Gly Thr Ser Thr Phe Thr
        115                 120                 125

Gln Tyr Phe Ser Val Arg Glu Ser Thr Arg Thr Ser Gly Thr Val Thr
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130 135 140

Val Ala Asn His Phe Asn Phe Trp Ala Gln His Gly Phe Gly Asn Ser
145 150 155 160

Asp Phe Asn Tyr Gln Val Met Ala Val Glu Ala Trp Ser Gly Ala Gly
165 170 175

Ser Ala Ser Val Thr Ile Ser Ser
180

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Phe Thr Tyr Asp Glu Ser Ala Gly Thr Phe Ser Met Tyr Trp Glu Asp
20 25 30

Gly Val Ser Ser Asp Phe Val Val Gly Leu Gly Gly Trp Thr Thr Gly
35 40 45

Ser Ser Asn Ala Ile Thr Tyr Ser Ala Glu Tyr Ser Ala Ser Gly Ser
50 55 60

Ala Ser Tyr Leu Ala Val Tyr Gly Trp Val Asn Tyr Pro Gln Ala Glu
65 70 75 80

Tyr Tyr Ile Val Glu Asp Tyr Gly Asp Tyr Asn Pro Cys Ser Ser Ala
85 90 95

Thr Ser Leu Gly Thr Val Tyr Ser Asp Gly Ser Thr Tyr Gln Val Cys
100 105 110

Thr Asp Thr Arg Ile Asn Glu Pro Ser Ile Thr Gly Thr Ser Thr Phe
115 120 125

Thr Gln Tyr Phe Ser Val Arg Glu Ser Thr Arg Thr Ser Gly Thr Val
130 135 140

Thr Val Ala Asn His Phe Asn Phe Trp Ala His His Gly Phe His Asn
145 150 155 160

Ser Asp Phe Asn Tyr Gln Val Val Ala Val Glu Ala Trp Ser Gly Ala
165 170 175

Gly Ser Ala Ala Val Thr Ile Ser Ser

180 185

<210> 3
<211> 185
<212> PRT
<213> Bacillus circulans

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Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Ile Val
1 5 10 15

Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
20 25 30

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
35 40 45

Arg Thr Ile Asn Tyr Asn Ala Gly Val Trp Ala Pro Asn Gly Asn Gly
50 55 60

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
65 70 75 80

Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
85 90 95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr Arg
100 105 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
115 120 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
130 135 140

Thr Phe Thr Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
145 150 155 160

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
165 170 175

Ser Gly Ser Ser Asn Val Thr Val Trp
180 185

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<213> Bacillus pumilus

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Arg Thr Ile Thr Asn Asn Glu Met Gly Asn His Ser Gly Tyr Asp Tyr
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Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ser Met Thr Leu Asn Asn Gly
20 25 30

Gly Ala Phe Ser Ala Gly Trp Asn Asn Ile Gly Asn Ala Leu Phe Arg
35 40 45

Lys Gly Lys Lys Phe Asp Ser Thr Arg Thr His His Gln Leu Gly Asn
50 55 60

Ile Ser Ile Asn Tyr Asn Ala Ser Phe Asn Pro Ser Gly Asn Ser Tyr
65 70 75 80

Leu Cys Val Tyr Gly Trp Thr Gln Ser Pro Leu Ala Glu Tyr Tyr Ile
85 90 95

Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Ala Tyr Lys Gly Ser
100 105 110

Phe Tyr Ala Asp Gly Gly Thr Tyr Asp Ile Tyr Glu Thr Thr Arg Val
115 120 125

Asn Gln Pro Ser Ile Ile Gly Ile Ala Thr Phe Lys Gln Tyr Trp Ser
130 135 140

Val Arg Gln Thr Lys Arg Thr Ser Gly Thr Val Ser Val Ser Ala His
145 150 155 160

Phe Arg Lys Trp Glu Ser Leu Gly Met Pro Met Gly Lys Met Tyr Glu
165 170 175

Thr Ala Phe Thr Val Glu Gly Tyr Gln Ser Ser Gly Ser Ala Asn Val
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Met Thr Asn Gln Leu Phe Ile Gly Asn
195 200

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<212> PRT
<213> Bacillus subtilis

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Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
20 25 30

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
35 40 45

Arg Thr Ile Asn Tyr Asn Ala Gly Val Trp Ala Pro Asn Gly Asn Gly
50 55 60

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
65 70 75 80

Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
85 90 95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr Arg
100 105 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
115 120 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
130 135 140

Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
145 150 155 160

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
165 170 175

Ser Gly Ser Ser Asn Val Thr Val Trp
180 185

<210> 6
<211> 211
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<213> Clostridium acetobutylicum

<400> 6
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Ile Gly Val Asn Gly Gly Tyr Asp Tyr Glu Leu Trp Lys Asp Tyr Gly
20 25 30

Asn Thr Ser Met Thr Leu Lys Asn Gly Gly Ala Phe Ser Cys Gln Trp
35 40 45

Ser Asn Ile Gly Asn Ala Leu Phe Arg Lys Gly Lys Lys Phe Asn Asp
50 55 60

Thr Gln Thr Tyr Lys Gln Leu Gly Asn Ile Ser Val Asn Tyr Asn Cys
65 70 75 80

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Asn Tyr Gln Pro Tyr Gly Asn Ser Tyr Leu Cys Val Tyr Gly Trp Thr
                85                  90                  95

Ser Ser Pro Leu Val Glu Tyr Tyr Ile Val Asp Ser Trp Gly Ser Trp
            100                 105                 110

Arg Pro Pro Gly Gly Thr Ser Lys Gly Thr Ile Thr Val Asp Gly Gly
        115                 120                 125

Ile Tyr Asp Ile Tyr Glu Thr Thr Arg Ile Asn Gln Pro Ser Ile Gln
    130                 135                 140

Gly Asn Thr Thr Phe Lys Gln Tyr Trp Ser Val Arg Arg Thr Lys Arg
145                 150                 155                 160

Thr Ser Gly Thr Ile Ser Val Ser Lys His Phe Ala Ala Trp Glu Ser
                165                 170                 175

Lys Gly Met Pro Leu Gly Lys Met His Glu Thr Ala Phe Asn Ile Glu
            180                 185                 190

Gly Tyr Gln Ser Ser Gly Lys Ala Asp Val Asn Ser Met Ser Ile Asn
        195                 200                 205

Ile Gly Lys
    210


<210> 7
<211> 206
<212> PRT
<213> Clostridium stercorarium

<400> 7
Gly Arg Ile Ile Tyr Asp Asn Glu Thr Gly Thr His Gly Gly Tyr Asp
  1               5                  10                  15

Tyr Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ile Met Glu Leu Asn Asp
            20                  25                  30

Gly Gly Thr Phe Ser Cys Gln Trp Ser Asn Ile Gly Asn Ala Leu Phe
        35                  40                  45

Arg Lys Gly Arg Lys Phe Asn Ser Asp Lys Thr Tyr Gln Glu Leu Gly
    50                  55                  60

Asp Ile Val Val Glu Tyr Gly Cys Asp Tyr Asn Pro Asn Gly Asn Ser
65                  70                  75                  80

Tyr Leu Cys Val Tyr Gly Trp Thr Arg Asn Phe Leu Val Glu Tyr Tyr
                85                  90                  95
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Ile Val Glu Ser Trp Gly Ser Trp Arg Pro Pro Gly Ala Thr Pro Lys
100 105 110

Gly Thr Ile Thr Gln Trp Met Ala Gly Thr Tyr Glu Ile Tyr Glu Thr
115 120 125

Thr Arg Val Asn Gln Pro Ser Ile Asp Gly Thr Ala Thr Phe Gln Gln
130 135 140

Tyr Trp Ser Val Arg Thr Ser Lys Arg Thr Ser Gly Thr Ile Ser Val
145 150 155 160

Thr Glu His Phe Lys Gln Trp Glu Arg Met Gly Met Arg Met Gly Lys
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Ala Asn Val Tyr Lys Asn Glu Ile Arg Ile Gly Ala Asn Pro
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<210> 8
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<212> PRT
<213> Ruminococcus flavefaciens

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20 25 30

Ala Gly Ser Phe Thr Cys Ser Trp Ser Asn Ile Glu Asn Phe Leu Ala
35 40 45

Arg Met Gly Lys Asn Tyr Asp Ser Gln Lys Lys Asn Tyr Lys Ala Phe
50 55 60

Gly Asn Ile Val Leu Thr Tyr Asp Val Glu Tyr Thr Pro Arg Gly Asn
65 70 75 80

Ser Tyr Met Cys Val Tyr Gly Trp Thr Arg Asn Pro Leu Met Glu Tyr
85 90 95

Tyr Ile Val Glu Gly Trp Gly Asp Trp Arg Pro Pro Gly Asn Asp Gly
100 105 110

Glu Val Lys Gly Thr Val Ser Ala Asn Gly Asn Thr Tyr Asp Ile Arg
115 120 125

Lys Thr Met Arg Tyr Asn Gln Pro Ser Leu Asp Gly Thr Ala Thr Phe
130 135 140

Pro Gln Tyr Trp Ser Val Arg Gln Thr Ser Gly Ser Ala Asn Asn Gln
145 150 155 160

Thr Asn Tyr Met Lys Gly Thr Ile Asp Val Ser Lys His Phe Asp Ala
165 170 175

Trp Ser Ala Ala Gly Leu Asp Met Ser Gly Thr Leu Tyr Glu Val Ser
180 185 190

Leu Asn Ile Glu Gly Tyr Arg Ser Asn Gly Ser Ala Asn Val Lys Ser
195 200 205

Val Ser Val
210

<210> 9
<211> 197
<212> PRT
<213> Schizophyllum commune

<400> 9
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1 5 10 15

Trp Trp Thr Asp Gly Ala Gly Asp Ala Thr Tyr Gln Asn Asn Gly Gly
20 25 30

Gly Ser Tyr Thr Leu Thr Trp Ser Gly Asn Asn Gly Asn Leu Val Gly
35 40 45

Gly Lys Gly Trp Asn Pro Gly Ala Ala Ser Arg Ser Ile Ser Tyr Ser
50 55 60

Gly Thr Tyr Gln Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp
65 70 75 80

Thr Arg Ser Ser Leu Ile Glu Tyr Tyr Ile Val Glu Ser Tyr Gly Ser
85 90 95

Tyr Asp Pro Ser Ser Ala Ala Ser His Lys Gly Ser Val Thr Cys Asn
100 105 110

Gly Ala Thr Tyr Asp Ile Leu Ser Thr Trp Arg Tyr Asn Ala Pro Ser
115 120 125

Ile Asp Gly Thr Gln Thr Phe Glu Gln Phe Trp Ser Val Arg Asn Pro
130 135 140

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Lys Lys Ala Pro Gly Gly Ser Ile Ser Gly Thr Val Asp Val Gln Cys
145                 150                 155                 160

His Phe Asp Ala Trp Lys Gly Leu Gly Met Asn Leu Gly Ser Glu His
                165                 170                 175

Asn Tyr Gln Ile Val Ala Thr Glu Gly Tyr Gln Ser Ser Gly Thr Ala
            180                 185                 190

Thr Ile Thr Val Thr
        195


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<212> PRT
<213> Streptomyces lividans

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  1               5                  10                  15

Tyr Ser Phe Trp Thr Asp Ser Gln Gly Thr Val Ser Met Asn Met Gly
             20                  25                  30

Ser Gly Gly Gln Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val
         35                  40                  45

Ala Gly Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Gln Tyr Ser
     50                  55                  60

Gly Ser Phe Asn Pro Ser Gly Asn Ala Tyr Leu Ala Leu Tyr Gly Trp
 65                  70                  75                  80

Thr Ser Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Thr
                 85                  90                  95

Tyr Arg Pro Thr Gly Glu Tyr Lys Gly Thr Val Thr Ser Asp Gly Gly
            100                 105                 110

Thr Tyr Asp Ile Tyr Lys Thr Thr Arg Val Asn Lys Pro Ser Val Glu
        115                 120                 125

Gly Thr Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg
    130                 135                 140

Thr Gly Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
145                 150                 155                 160

Ala Gly Met Pro Leu Gly Asn Phe Ser Tyr Tyr Met Ile Asn Ala Thr
                165                 170                 175
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Glu Gly Tyr Gln Ser Ser Gly Thr Ser Ser Ile Asn Val Gly Gly
180 185 190

<210> 11
<211> 191
<212> PRT
<213> Streptomyces lividans

<400> 11
Ala Thr Thr Ile Thr Thr Asn Gln Thr Gly Thr Asp Gly Met Tyr Tyr
1 5 10 15

Ser Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly
20 25 30

Gly Gly Ser Tyr Ser Thr Gln Trp Thr Asn Cys Gly Asn Phe Val Ala
35 40 45

Gly Lys Gly Trp Ser Thr Gly Asp Gly Asn Val Arg Tyr Asn Gly Tyr
50 55 60

Phe Asn Pro Val Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
85 90 95

Pro Thr Gly Thr Tyr Lys Gly Thr Val Ser Ser Asp Gly Gly Thr Tyr
100 105 110

Asp Ile Tyr Gln Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Gly Thr
115 120 125

Lys Thr Phe Gln Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
130 135 140

Gly Ser Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
145 150 155 160

Ala Gly Met Asn Met Gly Gln Phe Arg Tyr Tyr Met Ile Asn Ala Thr
165 170 175

Glu Gly Tyr Gln Ser Ser Gly Ser Ser Asn Ile Thr Val Ser Gly
180 185 190

<210> 12
<211> 189
<212> PRT
<213> Streptomyces sp.

<400> 12
Ala Thr Thr Ile Thr Asn Glu Thr Gly Tyr Asp Gly Met Tyr Tyr Ser
1   5   10   15

Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly Gly
            20   25   30

Gly Ser Tyr Ser Thr Arg Trp Thr Asn Cys Gly Asn Phe Val Ala Gly
        35   40   45

Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Arg Tyr Thr Gly Trp
    50   55   60

Phe Asn Pro Ser Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
65   70   75   80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
                85   90   95

Pro Thr Gly Glu Thr Arg Gly Thr Val His Ser Asp Gly Gly Thr Tyr
            100   105   110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Ala Pro
        115   120   125

Ala Ala Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
    130   135   140

Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg Ala Gly
145   150   155   160

Met Asn Met Gly Asn Phe Arg Tyr Tyr Met Ile Asn Ala Thr Glu Gly
                165   170   175

Tyr Gln Ser Ser Gly Ser Ser Thr Ile Thr Val Ser Gly
            180   185

<210> 13
<211> 189
<212> PRT
<213> Thermomonospora fusca

<400> 13
Ala Val Thr Ser Asn Glu Thr Gly Tyr His Asp Gly Tyr Phe Tyr Ser
1   5   10   15

Phe Trp Thr Asp Ala Pro Gly Thr Val Ser Met Glu Leu Gly Pro Gly
            20   25   30

Gly Asn Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val Ala Gly
        35   40   45

Lys Gly Trp Ala Thr Gly Gly Arg Arg Thr Val Thr Tyr Ser Ala Ser
50 55 60

Phe Asn Pro Ser Gly Asn Ala Tyr Leu Thr Leu Tyr Gly Trp Thr Arg
65 70 75 80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Glu Ser Trp Gly Thr Tyr Arg
85 90 95

Pro Thr Gly Thr Tyr Met Gly Thr Val Thr Thr Asp Gly Gly Thr Tyr
100 105 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Ile Glu Gly Thr
115 120 125

Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg Thr Ser
130 135 140

Gly Thr Ile Thr Ala Gly Asn His Phe Asp Ala Trp Ala Arg His Gly
145 150 155 160

Met His Leu Gly Thr His Asp Tyr Met Ile Met Ala Thr Glu Gly Tyr
165 170 175

Gln Ser Ser Gly Ser Ser Asn Val Thr Leu Gly Thr Ser
180 185

<210> 14
<211> 190
<212> PRT
<213> Trichoderma harzianum

<400> 14
Gln Thr Ile Gly Pro Gly Thr Gly Tyr Ser Asn Gly Tyr Tyr Tyr Ser
1 5 10 15

Tyr Trp Asn Asp Gly His Ala Gly Val Thr Tyr Thr Asn Gly Gly Gly
20 25 30

Gly Ser Phe Thr Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Ile Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145 150 155 160

Ser His Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
165 170 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
180 185 190

<210> 15
<211> 178
<212> PRT
<213> Trichoderma reesei

<400> 15
Ala Ser Ile Asn Tyr Asp Gln Asn Tyr Gln Thr Gly Gly Gln Val Ser
1 5 10 15

Tyr Ser Pro Ser Asn Thr Gly Phe Ser Val Asn Trp Asn Thr Gln Asp
20 25 30

Asp Phe Val Val Gly Val Gly Trp Thr Thr Gly Ser Ser Ala Pro Ile
35 40 45

Asn Phe Gly Gly Ser Phe Ser Val Asn Ser Gly Thr Gly Leu Leu Ser
50 55 60

Val Tyr Gly Trp Ser Thr Asn Pro Leu Val Glu Tyr Tyr Ile Met Glu
65 70 75 80

Asp Asn His Asn Tyr Pro Ala Gln Gly Thr Val Lys Gly Thr Val Thr
85 90 95

Ser Asp Gly Ala Thr Tyr Thr Ile Trp Glu Asn Thr Arg Val Asn Glu
100 105 110

Pro Ser Ile Gln Gly Thr Ala Thr Phe Asn Gln Tyr Ile Ser Val Arg
115 120 125

Asn Ser Pro Arg Thr Ser Gly Thr Val Thr Val Gln Asn His Phe Asn
130 135 140

Trp Ala Ser Leu Gly Leu His Leu Gly Gln Met Met Asn Tyr Gln Val
145 150 155 160

Val Ala Val Glu Gly Trp Gly Gly Ser Gly Ser Ala Ser Gln Ser Val
165 170 175

Ser Asn

<210> 16
<211> 190
<212> PRT
<213> Trichoderma reesei

<400> 16
Gln Thr Ile Gln Pro Gly Thr Gly Tyr Asn Asn Gly Tyr Phe Tyr Ser
1 5 10 15

Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
20 25 30

Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145 150 155 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
165 170 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
180 185 190

<210> 17
<211> 190
<212> PRT
<213> Trichoderma viride

<400> 17
Gln Thr Ile Gln Pro Gly Thr Gly Phe Asn Asn Gly Tyr Phe Tyr Ser
1 5 10 15

Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
20 25 30

Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Thr His
130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145 150 155 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
165 170 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
180 185 190

<210> 18
<211> 596
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX synthetic
sequence

<400> 18
ctagctaagg aggctgcaga tgcaaacaat acaaccagga accggttaca acaacggtta 60
cttttacagc tattggaacg atggccatgg tggtgttacc tatacaaacg ggcccggagg 120
ccaatttagc gtcaattggt ctaactccgg aaacttcgta ggtggaaaag gttggcaacc 180
cgggaccaaa aataaggtga tcaacttctc tggatcttat aatccgaatg ggaattcata 240
cttaagcgtc tatggctggt ctagaaaccc actgattgaa tattacattg tcgaaaattt 300
cggtacctac aatccgagta ccggcgccac aaaattaggc gaagtcacta gtgatggatc 360
cgtatatgat atctaccgta cccaacgcgt taatcagcca tcgatcattg gaaccgccac 420
cttttatcag tactggagtg ttagacgtaa tcatcggagc tccggttcgg ttaatactgc 480
gaatcacttt aatgcatggg cacagcaagg gttaacccta ggtacaatgg attatcaaat 540
cgtagcggtg gaaggctact tctcgagtgg ttccgctagt attacagtga gctaaa 596

<210> 19
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Trx-110C
Synthetic Sequence

<400> 19
atatacggat ccatcacaag tgacttcgcc taattttgtg 40

<210> 20
<211> 68
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-110C-2

<400> 20
gcgccacaaa attaggcgaa gtcacttgtg atggatccgt atatgatatc taccgtaccc 60
aacgcgtt 68

<210> 21
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-103b

<400> 21
aatcagccat cgatcattgg aaccgccacc ttttatcagt ac 42

<210> 22

<211> 54
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-109
Synthetic sequence

<400> 22
ggtggcggtt ccaatgatcg atggctgatt aacgcgttgg gtacggtaga tatc 54

<210> 23
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-108b

<400> 23
cgaaccggag ctccgatgat tacgtctaac actccagtac tgataaaa 48

<210> 24
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-154C
Synthetic sequence

<400> 24
ctagggttaa cccttgtgat gcccaggcat taaagtggca tgcagtatta ac 52

<210> 25
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-154C-2

<400> 25
tggagtgtta gacgtaatca tcggagctcc ggttcggtta atactgcatg ccactttaat 60
gcctgggcac agcaagggtt aacc 84

<210> 26
<211> 34

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-162H-3

<400> 26
ccacttcaat gcatgggcac agcacgggtt aacc 34

<210> 27
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-162H-4

<400> 27
ctagggttaa cccgtgctgt gcccatgcat tgaagtggca tg 42

<210> 28
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-101

<400> 28
tcgacaattt cggtacctac aatccgagta ccggcgccac aaaattaggc gaagtcac 58

<210> 29
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-102

<400> 29
tagtgatgga tccgtatatg atatctaccg tacccaacgc gttaatcagc ca 52

<210> 30
<211> 60
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:TrX-103

<400> 30
tcgatcattg gaaccgccac cttttatcag tactggagtg ttagacgtaa tcatcggagc 60

<210> 31
<211> 69
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-104

<400> 31
tccggttcgg ttaatactgc gaatcacttt aatgcatggg cacagcaagg gttaacccta 60
ggtacaatg 69

<210> 32
<211> 67
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-105

<400> 32
gattatcaaa tcgtagcggt ggaaggctac ttctcgagtg gttccgctag tattacagtg 60
agctaaa 67

<210> 33
<211> 53
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-106
synthetic sequence

<400> 33
gatctttagc tcactgtaat actagcggaa ccactcgaga agtagccttc cac 53

<210> 34
<211> 66
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-107

<400> 34
cgctacgatt tgataatcca ttgtacctag ggttaaccct tgctgtgccc atgcattaaa 60
gtgatt 66

<210> 35
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-108

<400> 35
cgcagtatta accgaaccgg agctccgatg attacgtcta acactccagt actgataaaa 60

<210> 36
<211> 73
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-110

<400> 36
atatacggat ccatcactag tgacttcgcc taattttgtg gcgccggtac tcggattgta 60
ggtaccgaaa ttg 73

<210> 37
<211> 76
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-1

<400> 37
ctagctaagg aggctgcaga tgcaaacaat acaaccagga accggttaca acaacggtta 60
cttttacagc tattgg 76

<210> 38
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-2

<400> 38

aacgatggcc atggtggtgt tacctataca aacgggcccg gaggccaatt tagcgtcaat 60
tggtctaact ccggaaac 78

<210> 39
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-3

<400> 39
ttcgtaggtg gaaaaggttg gcaacccggg accaaaaata aggtgatcaa cttctctgga 60
tcttataatc cgaatggg 78

<210> 40
<211> 74
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-4

<400> 40
aattcatact taagcgtcta tggctggtct agaaacccac tgattgaata ttacattgtc 60
gaaaatttcg gtac 74

<210> 41
<211> 85
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-5

<400> 41
gcaaattttc gacaatgtaa tattcaatca gtgggtttct agaccagcca tagacgctta 60
agtatgaatt cccattcgga ttata 85

<210> 42
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Trx-6Synthetic
sequence

<400> 42
agatccagag aagttgatca ccttattttt ggtcccgggt tgccaacctt ttccacctac 60
gaagtttccg gagttaga 78

<210> 43
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:XyTv-7
Synthetic sequence

<400> 43
ccaattgacg ctaaattggc ctccgggccc gtttgtatag gtaacaccac catggccatc 60
gttccaatag ctgtaaaagt aacc 84

<210> 44
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-8 synthetic
sequence

<400> 44
gttgttgtaa ccggttcctg gttgtattgt ttgcatctgc agcctcctta g 51

<210> 45
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-108C
synthetic sequence

<400> 45
atatacggat ccatcactag tgcattcgcc taattttgtg 40

<210> 46
<211> 68
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-108C-2

<400> 46
gcgccacaaa attaggcgaa tgcactagtg atggatccgt atatgatatc taccgtaccc 60
aacgcgtt 68

<210> 47
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-158C-162H
synthetic sequence

<400> 47
ctagggttaa cccgtgtgat gcccagcaat taaagtgatt tgcagtatta ac 52

<210> 48
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-158C-162H-2

<400> 48
tggagtgtta gacgtaatca tcggagctcc ggttcggtta atactgcaaa tcactttaat 60
tgctgggcac agcacgggtt aacc 84

<210> 49
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-108C-110C
synthetic seqeuence

<400> 49
atatacggat ccatcacaag tgcattcgcc taattttgtg 40

<210> 50
<211> 68
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Tx-108C-110C-2
synthetic sequence

<400> 50
gcgccacaaa attaggcgaa tgcacttgtg atggatccgt atatgatatc taccgtaccc 60
aacgcgtt 68

<210> 51
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial
Sequence:Tx-154C-158C-152H synthetic seqeunce

<400> 51
ctagggttaa cccgtgtgat gcccagcaat taaagtggca tgcagtatta ac 52

<210> 52
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial
Sequence:Tx-154C-158C-162H-2

<400> 52
tggagtgtta gacgtaatca tcggagctcc ggttcggtta atactgcatg ccactttaat 60
tgctgggcac agcacgggtt aacc 84

<210> 53
<211> 190
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX amino acid
sequence

<400> 53
Gln Thr Ile Gln Pro Gly Thr Gly Tyr Asn Asn Gly Tyr Phe Tyr Ser
1 5 10 15

Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
20 25 30

Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly

50 55 60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65 70 75 80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
100 105 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
115 120 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
130 135 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145 150 155 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
165 170 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
180 185 190

<210> 54
<211> 198
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-DS1
cassette

<400> 54
gcgccacaaa attaggcgaa gtcacttgtg atggatccgt atatgatatc taccgtaccc 60
aacgcgttaa tcagccatcg atcattggaa ccgccacctt ttatcagtac tggagtgtta 120
gacgtaatca tcggagctcc ggttcggtta atactgcatg ccactttaat gcctgggcac 180
agcaagggtt aaccctag 198

<210> 55
<211> 67
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-DS1
cassette aa

<400> 55
Gly Ala Thr Lys Leu Gly Glu Val Thr Cys Asp Gly Ser Val Tyr Asp
1 5 10 15

Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile Ile Gly Thr Ala
20 25 30

Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His Arg Ser Ser Gly
35 40 45

Ser Val Asn Thr Ala Cys His Phe Asn Ala Trp Ala Gln Gln Gly Leu
50 55 60

Thr Leu Gly
65

<210> 56
<211> 15
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-162H-DS1
cassette aa

<400> 56
Ala Cys His Phe Asn Ala Trp Ala Gln His Gly Leu Thr Leu Gly
1 5 10 15

<210> 57
<211> 198
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-162H-DS2
cassette

<400> 57
gcgccacaaa attaggcgaa tgcactagtg atggatccgt atatgatatc taccgtaccc 60
aacgcgttaa tcagccatcg atcattggaa ccgccacctt ttatcagtac tggagtgtta 120
gacgtaatca tcggagctcc ggttcggtta atactgcaaa tcactttaat tgctgggcac 180
agcacgggtt aaccctag 198

<210> 58
<211> 67
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-162H-DS2
cassette aa

<400> 58
Gly Ala Thr Lys Leu Gly Glu Cys Thr Ser Asp Ser Ser Val Tyr Asp
1 5 10 15

Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile Ile Gly Thr Ala
20 25 30

Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His Arg Ser Ser Gly
35 40 45

Ser Val Asn Thr Ala Asn His Phe Asn Cys Trp Ala Gln His Gly Leu
50 55 60

Thr Leu Gly
65

<210> 59
<211> 198
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-162H-DS4
cassette

<400> 59
gcgccacaaa attaggcgaa tgcacttgtg atggatccgt atatgatatc taccgtaccc 60
aacgcgttaa tcagccatcg atcattggaa ccgccacctt ttatcagtac tggagtgtta 120
gacgtaatca tcggagctcc ggttcggtta atactgcatg ccactttaat tgctgggcac 180
agcacgggtt aaccctag 198

<210> 60
<211> 67
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-162H-DS4
cassete aa

<400> 60
Gly Ala Thr Lys Leu Gly Glu Cys Thr Cys Asp Gly Ser Val Tyr Asp
1 5 10 15

Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile Ile Gly Thr Ala
20 25 30

Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His Arg Ser Ser Gly
35 40 45

Ser Val Asn Thr Ala Cys His Phe Asn Cys Trp Ala Gln His Gly Leu
50 55 60

Thr Leu Gly
65

<210> 61
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:TrX-162H-DS1
cassette

<400> 61
catgccactt caatgcatgg gcacagcacg ggttaaccct ag 42